Novel aspects on the role of IFN-β in inflammation and immunity

Von dem Fachbereich für Biowissenschaften und Psychologie
der Technischen Universität Carola-Wilhelmina
zu Braunschweig
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr.rer.nat.)

genehmigte
D i s s e r t a t i o n

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eingereicht am: 31.03.2005
mündliche Prüfung (Disputation) am: 04.07.2005
Druckjahr: 2005

Vorveröffentlichungen der Dissertation

Teilergebnisse aus dieser Arbeit wurden mit Genehmigung der Gemeinsammen Naturwissenschaftlichen Fakultät, vertreten durch den Mentor der Arbeit, in folgenden Beiträgen vorab veröffentlicht:

Tagungsbeiträge:

Samuelsson C.V., zur Lage S., Weiss S. IFNβ supports an effective in vitro immune response (Poster), 18th Conference of the European Macrophage and Dendritic Cell Society, Barcelona, Spain (2004)

Loessner H, Samuelsson CV, Jablonska J, Lienenklaus S, Medina E, Weiss S Role of IFNβ in bacterial infection (Poster), Joint Annual Meeting of the German and Dutch Societies for Immunology, Maastricht, The Netherlands (2004**)**

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1 Introduction

The birth of immunology as a science is usually attributed to Edward Jenner, who in 1796 showed that cowpox induced protection against the human smallpox. Though, what actually caused diseases was not discovered until the late 19th century, when Robert Koch proved that infectious diseases are caused by microorganisms. Today, four broad categories of disease causing microorganisms are known: viruses, bacteria, pathogenic fungi and parasites. The immune system is designed to protect the host from these pathogenic microorganisms, and consists of two branches: innate and adaptive immunity.

1.1 The Immune System

The innate immune system is the body's first line of defence which detects and destroys microorganisms within minutes or hours after encountering them. Unlike the adaptive immune responses, which rely on clonal expansion of antigen specific lymphocytes, the innate immune response is very quick because it consists of a "finished" network of cells (e.g. phagocytes and NK cells) (Hamerman et al., 2005) and soluble factors (e.g. defensins, complement, lysozyme and cytokines) (Janeway, et al., 2002). The cells in the innate immune system react against different molecules that are more or less specific for pathogens. This recognition is achieved by invariant pattern recognition receptors (PRRs) on the host cell, which detect common features of pathogens called pathogen-associated molecular patterns (PAMPs), e.g. bacterial cell wall components like lipopolysaccharide (LPS) and lipoteichoic acid (LTA), as well as double- or single-stranded RNA and unmethylated bacterial DNA motifs (CpG DNA) (Theofilopoulos et al., 2004).
The first barrier that is encountered by pathogens are the epithelial layers that line the inner and outer surfaces of the body. These include skin, gastrointestinal tract, lungs, eyes and nose. These barriers preventing pathogens from colonizing tissues are either mechanical (e.g. tight junctions joining epithelial cells or movement of mucous by cilia) or chemical (e.g. low pH in the gut, enzymes and antibacterial peptides). If a pathogen still manages to cross the epithelial barrier it is often immediately recognized by phagocytes resident in the tissue. These innate responses induced during the initial phase of an infection do not generate an immunological memory, but help trigger the adaptive immune system.

Adaptive immunity, which is based on clonal selection and expansion from a repertoire of lymphocytes bearing diverse antigen-specific receptors, allows the immune system to

recognize virtually any foreign antigen. The two cellular mediators of adaptive immunity, the T and the B lymphocytes, have two distinct recognition systems for detection of pathogens. B cells have cell surface immunoglobulin molecules as receptors for direct antigen recognition, while the T cells have receptors that recognize peptide fragments processed and presented by infected cells. The specificities of these receptors are generated by somatic mechanisms and each individual lymphocyte carries receptors specific for only a single antigen, which is in contrast to the invariant PRRs of the innate immune system. After encountering and recognizing their specific antigen, the B and T cells undergo a clonal expansion stage that leads to the generation of many effector cells that can carry on surveying the host for more pathogens or pathogen specific peptides. In addition, this clonal expansion also leads to the generation of long lived cells that provide the host with an immunological memory protecting from reinfection. The reactivity of T and B cells is dependent on signals provided by the innate recognition system, and the synergistic actions of both the innate and adaptive immune responses provide the host with a rapid and highly specific protective response against microorganisms.

1.1.1 Cells of the Innate Immune System

Most of the cells of the innate immune system are phagocytic cells that can take up microorganisms by a wide variety of receptors, e.g. scavenger receptors and Fc-receptors. These cells develop in the bone marrow and belong to the same cell linage.

1.1.1.1 Macrophages

Macrophages, or mononuclear phagocytes, reside in tissues in close vicinity to the epithelial cell barriers, and are well suited to recognize any pathogen that breaks through this first barrier. They are mostly found in connective tisssue, in submucosal layers of the gastrointestinal tract, in the lungs and throughout the spleen. The macrophages continuosly mature from monocytes that leave the circulation to migrate into tissues throughout the body. Because of their location, macrophages are often the first cells to encounter pathogens and play an important role in innate immunity as antigen-presenting cells, but also as effector cells in humoral and cell-mediated immunity. The key functions of macrophages is to recognize infectious “nonself” and damaged “self”, and they phagocytose and degrade apoptotic cells, microbes and neoplastic cells. Furthermore, macrophages produce cytokines and other soluble mediators of inflammation and are also involved in antigen presentation and T-cell stimulation.

1.1.1.2 Dendritic cells

Dendritic cells (DCs) can be found in almost all non-lymphoid tissues, where they reside as immature DCs for a long time. Upon antigen uptake or cytokine triggering they proliferate and migrate into the lymph nodes and spleen, where they essentially transport the antigen to inductive sites.

Mouse DCs can be divided into several subpopulations depending on their cell surface markers or their location in the body: $CD8^+$, $CD8^-CD4^+$, $CD8^-CD4^-$, Langerhans cells, dermal DCs, $CD45^+$ plasmacytoid DCs (pDCs) (Shortman et al., 2002) and finally the newly charaterized TNF- and iNOS producing dendritic cells (TipDCs) (Serbina et al., 2003). The differences in function of theses individual subsets is still under investigation, but a few general notes can be made on DCs.

DCs are the most potent antigen presenting cells (APCs) in the host. They constitutively express both MHC-I and –II on their surface and can therefore present antigen to both CD4+ and CD8+ T cells. The DC membrane is also rich in costimulatory molecules that facilitate the binding of T cells to DCs, though the amounts of these molecules on the DC surface is dependent on the DC's maturation status. Immature DCs, that have yet to encounter a pathogen, express comparatively low levels of these molecules, and cannot stimulate T cells effectively. Mature DCs, on the other hand, have encountered a pathogen and are activated. They can very effectively activate T cell responses, though have no or very little phagocytic ability in comparison to immature DCs (Banchereau et al., 1998). In response to stimuli DCs also secrete cytokines, e.g. IL-1 and TNFα. Furthermore, it is speculated that CD8+ and CD8- DCs differ in their cytokine production pattern, and it has been suggested that CD8+ DCs are involved in the induction of T helper 1 responses by producing high levels of IL-12, while CD8- DCs produce low levels of IL-12 and therefore mainly induce T helper 2 responses (Ardavin, 2003).

pDCs are characterized by their potential to produce large amounts of type I IFN in response to virus infection (Asselin-Paturel et al., 2001). Furthermore, they have also been implicated to be involved in the maintenance of T-cell tolerance by inducing the differentiation of T regulatory cells (Martin et al., 2002).

1.1.1.3 Neutrophils

Neutrophilic granulocytes are also known as polymorphonuclear neutrophilic leukocytes and represent another major family of phagocytes. Mature neutrophils, which are abundant in

blood but absent in healthy tissue, are generally short lived cells with a half life of ½ day. The neutrophils play an important role in engulfing and killing extracellular pathogens and usually represent the earliest phogocytes to migrate into an inflamed site.
The neutrophils belong to a group of cells called granulocytes, which also include two other cell types called eosinophils and basophils. The eosinophils are mainly important in the defense against parasitic infections and can release aggressive compounds upon activation, while basophils have functions similar to, or complementary, to those of mast cells, and are responsible for attracting effector leukocytes to inflammatory sites (Bochner et al., 2001).

1.1.1.4 NK cells

NK cells develop in the bone marrow from the common lymphoid progenitor and circulate in the blood. They are activated by type I IFNs and other macrophage-derived cytokines (Biron, 1997; Orange et al., 1995; Pilaro et al., 1994), and serve as an early defense against intracellular infections. These large, granular cells are defined by their ability to kill certain tumor cells. They also play an important role in innate immunity to viruses and other intracellular pathogens, as well as in antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells have two roles during early stages of infection. The first is to kill infected cells directly, the second is to produce cytokines, e.g. IFNγ, that then influence the immune response through activation of other cell types.

1.1.2 Cells of the Adaptive Immune System

B and T lymphocytes are the effector cells of the adaptive immune system, and both have receptors specific for antigen. During ontogeny the receptor genes are assembled from gene segments by somatic rearrangement.

1.1.2.1 B cells

B cells are the cells responsible for humoral immunity and are derived from hematopoietic stem cells in the bone marrow, just like the T cells and other blood cells. They express immunoglobulins on their surfaces which can recognize native antigen. After antigen contact and activation the B cells can differentiate into antibody secreting plasma cells. Antibodies help combat an infection in different ways. First, they can neutralize bacterial toxins and viruses by binding to them and thereby block the pathogen's access to cells. Second, the antibodies coat pathogens and other foreign particles providing a signal for their elimination

by phagocytic cells of the innate immune system. Third, the complement system is activated upon antigen-binding, leading to destruction and removal of the pathogen.

1.1.2.2 T cells

The T lymphocytes, or T cells, are derived from hematopoietic stem cells and are responsible for cellular immunity. T cell progenitors migrate to the thymus from the fetal liver before birth, or from the bone marrow after birth. In the thymus, the T cells mature (Kruisbeek, 1999) whereafter they leave the thymus to recirculate through the secondary lymphoid organs (e.g. spleen, lymph nodes and Peyer's patches). The T cell receptor (TCR) is the antigen binding structure that is expressed on the T cell surface. In the majority of T cells, the TCR is a heterodimer consisting of an α and a β chain (α:β T cells). α:β T cells can not recognize native antigen but only peptide fragments bound to one of the major histocompatability complex (MHC) molecules. MHC class I molecules mainly present peptides derived from intracellular antigen, whereas MHC class II molecules primarily present peptides derived from proteins taken up via endocytic vesicles (Watts et al., 1999).

The α:β T cells can be divided into at least two subsets based on their cell surface markers and functional properties: cytotoxic T cells that carry CD8 on their surface and T-helper cells that are positive for CD4. $CD8^+$ cytotoxic cells can recognize infected cells through antigens presented on MHC-I. After recognition the cytotoxic T cells have the ability to directly kill the infected cell by inducing apoptosis and thus preventing further spreading of the virus.

The T-helper cells on the other hand, are specialized to activate other effector cells after having recognized peptide fragments presented by MHC-II, and can be further subdivided into T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cells activate macrophages to kill pathogens (Paulnock, 1992) and Th2 cells activate B cells to differentiate and to produce antibodies (Parker, 1993).

Recently a new subpopulation of α:β T cells, the regulatory T cells (T_{Reg}), has been described (Sakaguchi et al., 1995). T_{Reg} play a crucial role in allergic reactions, in suppressing immune responses to self-antigens and in preventing autoimmune diseases. Existing evidence also implies a role for regulatory T cells in immune responses to bacteria, viruses, parasites and fungi (Mills, 2004).

Another type of T cell carries an alternative form of the TCR. These γ:δ T cells are to date not well characterized and their exact functions and ligands are still unknown.

1.1.3 Innate immune recognition

In order for the host to mount a effective immune response against pathogens, the actions of the cells in the innate and adaptive immune systems need to be coordinated. This is accomplished by the release of cellular factors, called cytokines, that in auto- and paracrine manners activate or deactivate neighbouring cells. Before the cytokines can be produced and released though, immune cells, especially cells of the innate immune system, need to recognize pathogens and thus be activated by this pathogen recognition. The different ways this can be achieved will be reviewed on the following pages.

1.1.3.1 Phagocytosis

As already mentioned, cells of the innate immune system have the ability to ingest foreign particles and subsequently clear these. The ingestion of particles is facilitated by interactions between receptors on the phagocyte and ligands on the surfaces of particles, and is one of the key elements in host defense against bacterial infections.

Phagocytosis can be carried out in two different ways. One is opsonin independent, and ligands on the pathogen or particle is directly recognized by receptors on the phagocytic cell. Receptors for this mechanism will be discussed in more detail below. The second mechanism is opsonin dependent, meaning that the surface of a pathogen, or another particle, is altered by immunoglobulins or complement to facilitate the uptake into macrophages or neutrophils via Fcγ or complement receptors.

Three classes of Fcγ receptor (FcγR) have been found on human monocytes. FcγRI and FcγRII are constitutively expressed, while FcγRIII expression is low but can be induced on differentiating monocytes. The stimulation of monocytes through the FcγR activates phagocytosis, the production of cytokines and also the release of reactive oxygen species (Swanson et al., 2004).

Complement receptors are know to bind directly to certain viruses and bacteria and to facilitate the opsonization of these. This opsonization does not always trigger release of reactive oxygen species, but rather enhances the killing of microorganisms by neutrophils (Hart et al., 2004).

More important for this work are the opsonin independent receptors, which can broadly be divided into three groups: lectins, scavenger recptors and the toll-like receptors (TLRs). These all recognize particular PAMPs on the surfaces of microorganisms, or PAMPs derived from microrganisms.

1.1.3.2 Lectins

Lectins are expressed on macrophages, DCs and some endothelial cells. They specifically crosslink with carbohydrates found on a wide range of bacteria, fungi, parasites and virus infected cells. Their physiological function is still unclear, though lectins that recognize endogenous ligands seem to play a role in fertilization and development by facilitating cell-cell adhesion. Lectins that recognize exogenous ligands are divided into two groups. C-type lectins, or selectins, recognize mannose groups on bacteria and activate complement through the mannose-binding protein (McGreal et al., 2004; Nakagawa et al., 2003). They can also mediate adhesion of leukocytes to the endothelium, thus inducing rolling and facilitating the recruitment of leukocytes to sites of inflamed tissue (Graves et al., 1994). The second group is the S-type lectins, or galectins. They preferentially bind to glycoconjugates containing the ubiquitous disaccharide *N*-acetyllactosamine. Galectins have been shown to influence cell adhesion, chemotaxis and also cell proliferation and apoptosis (Rubinstein et al., 2004).

1.1.3.3 Scavenger receptors

The scavenger receptors (SR) are, just like the lectins, expressed on macrophages, DCs and some endothelial cells. They are involved in receptor-mediated endocytosis of polyanionic ligands, like light density lipoprotein (LDL), and also bind and internalise micro-organisms and their products, e.g. LTA, LPS and CpG DNA. Furthermore, SRs have also been shown to be involved in phagocytosis of apoptotic cells as well as in cell adhesion (Peiser et al., 2002).

1.1.3.4 Toll-like receptors

The Toll-like receptors (TLRs) are the mammalian homologues of an evolutionary conserved receptor system originally discovered in Drosophila melanogaster. In Drosophila this receptor is known as Toll and the is involved in embryonic development, as well as in defense against fungi and other microbes. In mammals this system is now known to play a key role in defense against infection, and the role of the TLRs is to activate phagocytes to respond to pathogens by secreting cytokines and chemokines. There are now at least 12 TLRs known in the mouse and at least 11 in humans (Janeway et al., 2002; Zhang et al., 2004). They are predominantly expressed on the cell surface, although a subset (TLR-7, -8 and -9 and in some cases TLR-3) are retained in intracellular compartments, and each is devoted to recognizing a distinct set of molecular patterns that are not normally found in mammals.

TLR-2 recognizes peptidoglycans and lipoteichoic acids, present on the membrane of gram-positive bacteria (Takeda et al., 2004a). TLR-2 forms hetereodimers together with TLR-1 or -

6. In addition, these dimers also recognize zymosan from yeast (Underhill et al., 1999) and GPI-linked proteins on parasites (Campos et al., 2001).
TLR-3 is important in detecting virus infection and binds double-stranded RNA (dsRNA) (Alexopoulou et al., 2001), that might be derived from virus infected cells or from the uptake of apoptotic cells (Schulz et al., 2005). It is one of the important mediators of IFN-β activation.
TLR-4 was the first TLR to be well characterized (Poltorak et al., 1998). It recognizes, together with CD14, LPS on gram-negative bacterial cell walls, though can also bind F-protein from certain viruses, and is also an inducer of IFN-β.
Flagellin is the ligand for TLR-5 (Hayashi et al., 2001), while TLR-9 recognizes unmethylated CpG DNA sequences derived from bacteria (Bauer et al., 2001; Hemmi et al., 2000), but also herpes-virus DNA.
Recently, the natural ligand for murine TLR-7 and human TLR-7 and TLR-8 was found to be single-stranded RNA (ssRNA) (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). Also TLRs 7-9 are capable of initiating type I IFN synthesis.
TLR-11 recognizes uropathogenic bacteria in mice (Zhang et al., 2004), while the TLR-10 ligand is to date still unknown.
The TLRs contain multiple leucine-rich repeats in their extracellular domain and a conserved intracellular Toll/IL-1 receptor domain (TIR). Receptor binding triggers signaling cascade involving myeloid differentiation factor 88 (MyD88), IL-1R associated kinase (IRAK) and TNFR-associated factor 6 (TRAF6). This leads to the activation of both the nuclear factor kappa B (NFκB) and Jun amino-terminal kinase (JNK) signaling pathways (O'Neill, 2002). In addition to NFκB and JNK, the TLRs also activate other signaling cascades, including p38, ERK and the interferon regulatory factor (IRF-3) pathways. Activation of TLRs lead to major changes in gene expression, e.g. induction of inflammatory cytokines like TNFα, IL-1, IL-6 and IL-12 (Aderem, 2001). Signaling important for the induction of type I IFN will be explained in more detail below.

1.1.3.5 Intracellular recognition systems

Some pathogens have the ability to quickly enter cells, therefore also intracellular recognition systems exist. As already mentioned a subset of TLRs are located in vesicles inside cells. Apart from these intracellular TLRs, the family of NOD proteins make up another intracellular recognition system, and consists of the NOD1 and NOD2 proteins. The NODs

associates with a protein kinase, RIP2, which in turn can activate NFkB and MAP kinase signaling pathways (Janeway et al., 2002). The full range of ligands recognized by the NODs is still unknown, but both NOD1 and NOD2 have been reported to activate NFkB in response to LPS (Inohara et al., 2001; Ogura et al., 2001).
Another intracellular recognition system is the retinoic acid inducible gene I (RIG-I), which was recently shown to be an essential regulator for dsRNA induced signalling of type I IFNs (Yoneyama et al., 2004).

1.1.4 Innate immune reactions

Micro-organisms that are recognized by one of the above mentioned receptors of the innate immune system initiate a plethora of signals and reactions within the cell which it infects or binds to. These reactions in general include the upregulation of antigen presenting capacity, the upregulation of cell adhesion molecules, the induction of low molecular weight effector molecules and cytokines.
In order for the T cells of the adaptive immunity to be activated they need to be presented with proteolytically degraded peptide fragments. These peptides act as antigens and are presented to the T cells on MHC molecules found on the surface of antigen presenting cells (APCs). When APCs are infected with a pathogen they generally upregulate the number of MHC molecules they have on their surface, thus increasing their antigen presenting capacity and ability to activate T cells. The binding of T cells to the APCs is also dependent upon cell adhesion molecules, e.g. ICAM-1 and VCAM, and co-stimulatory molecules, e.g. CD80 (B7.1) and CD86 (B7.2), being upregulated. This further increases the capacity of an infected APC to activate T cells.
Infection also triggers the release of low molecular weight effector molecules in neutrophils and macrophages. These include reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI), and are induced by the stimulation of particular enzymatic pathways leading to substrate reduction or oxidation.
Other low molecular weight effectors include prostaglandins (PGs) and leukotrienes (LTs). These are locally secreted, pro-inflammatory lipid mediators that are derived from phospholipase A_2 degradation of arachidonic acid. PGs are involved in inflammatory responses, apoptosis and cell proliferation and degradation. Similarly, LTs are of importance in inflammation and in allergic reactions.
Cytokines are small proteins released by various cells in response to an activating stimulus. They act in an auto- and paracrine manner, binding to high affinity cell surface receptors and

trigger signalling pathways that lead to changes in gene expression. Under natural conditions, cells never encounter one cytokine at a time. Rather, cells are surrounded by a mixture of several cytokines which together act in synergistic and antagonistic ways that form the cell's outcoming biological action. Among the most important inflammatory cytokines released by cells in the innate immune response are IL-1, IL-6, IL-12, TNF-α and the type I IFNs. As this work is about type I IFNs they have been handed a section of their own.

1.2 The Interferon System

The IFNs are comprised of viral or type I IFNs (mainly α, β, ω and τ) and immune or type II IFN (γ). The two IFN types signal through distinct receptors. In addition, the novel IFN-λ sometimes is designated as a type III IFN (Oritani et al., 2004), as it does not share receptor with the other IFNs, but is most commonly sorted in under the type I IFNs because of its biological activity.

The IFNs were originally characterized in the late 1950s based on their antiviral properties (Isaacs et al., 1957), and have since been determined to play an essential role in protecting the host from viral infection (Barchet et al., 2002; Deonarain et al., 2000; Isaacs et al., 1957; Muller et al., 1994; van den Broek et al., 1995a; van den Broek et al., 1995b). However, accumulating studies now indicate that this view of IFN function is incomplete as it is clear that they also play major roles in innate anti-microbial defence (Bogdan, 2000) and may facilitate viral immune evasion (Hahm et al., 2005), apart from their established pleiotropic roles as immunomodulatory, antiproliferative or pro-apoptotic factors. In addition, influences of type I IFN-mediated signalling on bone development and differentiation of myeloid cells have also been reported (Bogdan, 2000; Deonarain et al., 2003; Takaoka et al., 2003b).

1.2.1 Subtypes of IFN

Type I IFN

Type I IFNs constitute a multi-member cytokine family consisting of IFN-α subtypes, -β, -ε, -κ, -ω, -δ, -τ, -λ and limitin (or -ξ). They are almost all intronless, IFN-κ and -λ being the exceptions, but have secretory signal peptide sequences that are cleaved prior to secretion. In humans there exists at least 14 non-allelic IFN-αs (including 5 pseudo genes) and at least 6 ω (including 5 pseudo genes) (Adolf, 1995), while IFN-β, -κ (Nardelli et al., 2002) and -ε are represented by a single member. These are all arranged in a genomic cluster on the short arm of chromosome 9 (De Maeyer et al., 1998; Diaz et al., 1994; Hardy et al., 2004; Roberts et al.,

1998). The murine type I IFN locus is located on chromosome 4 and contains 14 IFN-α genes, single IFN-β, -κ and -ε genes, and an undefined number of limitin genes (De Maeyer et al., 1998; Hardy et al., 2004; Kelley et al., 1985; Takahashi et al., 2001; van, V et al., 2004; Vassileva et al., 2003).

Of the other type I IFNs, IFN-δ and -τ are not found in humans but in pigs and in ruminant animals respectively, and so far have unspecified functions.

IFN-ε is expressed in the placenta of humans and might play a role in reproduction.

IFN-κ is predominantly expressed in keratinocytes of the skin (LaFleur et al., 2001) and responds to similar stimuli and also signals through the common type I IFN receptor.

Limitin has so far only been characterized in the mouse (Oritani et al., 2000; Oritani et al., 2003) and has similar effects as IFN-α and -β, though shows no or less severe myelosuppressive properties (Takahashi et al., 2001).

The last group of type I IFNs to have been identified is the IFN-λs, consisting of λ1 (IL-29), λ2 and λ3 (IL-28A and -B) (Kotenko et al., 2003; Sheppard et al., 2003). Though they are functionally similar to the other type I IFNs, their genes have distinct sequences and chromosomal locations. Moreover, the IFN-λs have several introns and don't signal through the common type I IFN receptor (IFNAR), even though they also induce activation of STAT-1, -2, -3, -5 and promote ISGF3 complex formation (Dumoutier et al., 2003; Dumoutier et al., 2004).

Type II IFN

To date IFN-γ is the only type II IFN indentified. It functions as an asymmetrical homodimer and binds to the extracellular domain of the type II IFN receptor complex (Kalvakolanu, 2003; Sen, 2001; Stark et al., 1998), the two subunits of which are called IFNGR1 and IFNGR2. IFN-γ is mainly produced by T cells and NK cells, and mediates antigen-specific immune responses. Furthermore, IFN-γ can upregulate the expression of MHC-II, activate macrophages for tumoricidal and bacteriocidal functions, promote differentiation of B and T cells, as well as activate NK cells.

Of the type I IFNs this work is mostly concerned with IFN-α and -β and the rest of this section will therefore focus on these two IFNs.

1.2.2 Cellular sources of type I IFN

Probably any cell can produce type I IFN in response to an appropriate viral stimulus *in vitro* and *in vivo*. Though within the immune system, monocytes and macrophages (Eloranta et al., 1999; Fleit et al., 1981), neutrophils (Shirafuji et al., 1990), conventional DCs (Diebold et al., 2003; Eloranta et al., 1997; Hochrein et al., 2001), pDCs (Colonna et al., 2002), NK cells (Peter et al., 1980), and T cells (Conta et al., 1983) have been shown to release type I IFN in response to mitogenic, viral and/or microbial stimuli. The most potent producers of type I IFN are the natural interferon producing cells (NIPC) found in human peripheral blood (Ronnblom et al., 1983), and their murine counterpart pDCs. These pDCs circulate as immature cells and can differentiate into potent antigen presenting cells after activation. In response to virus they produce large amounts of type I IFN in both the human and murine systems (Asselin-Paturel et al., 2001; Hochrein et al., 2002; Kadowaki et al., 2000) and they predominantly express TLR-7 and -9 as opposed to conventional DCs that have a broader spectrum of TLRs expressed (Colonna et al., 2002).

1.2.3 Induction and hierarchy of type I IFN

Infection by microorganisms (viruses, bacteria, protozoa, mycobacteria) is not the only way to induce type I IFN production in various cell types. Also, exposure to certain cytokines and growth factors (CSF-1, IL-1, IL-2 and TNFα) can induce the type I IFNs. E.g. stimulation of bone marrow derived macrophages (BMMϕ) with CSF-1 or IL-2 results in the production of murine IFN-α/β (Moore et al., 1984) and in human diploid fibroblasts TNFα and IL-1 induces IFN-β (Reis et al., 1989). Furthermore, IFNγ induces IFN-α/β in macrophages (Cantell et al., 1996; Gessani et al., 1989) and RANK ligand, a member of the TNF family, can also induce IFN-β in BMDMs (Taniguchi et al., 2002).

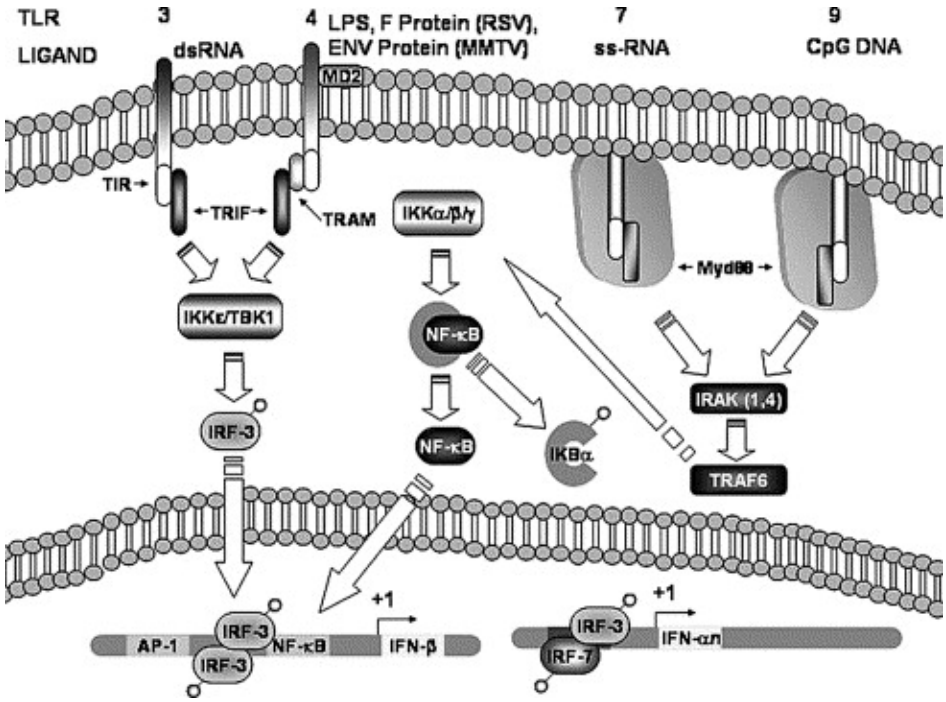


Fig. 1.1 Type I IFN induction pathways*. Illustrated are two of the most important type I IFN induction pathways in response to different PAMPs, with the most important adaptor and effector molecules.*

The signaling pathways leading to type I IFN expression have been most extensively studied in the context of TLR stimulation. Only TLR-3, -4, -7 and -9 have been shown to induce type I IFN induction upon ligand binding (summarized in Fig. 1.1). In order to transduce their signal, the TLRs interact with different adaptor molecules through their Toll-interleukin receptor (TIR) domain. MyD88 is the most prevalent adaptor and can interact with all TLRs (Akira et al., 2003). Binding of the TIR domains of MyD88 and a TLR initiates a signal transduction pathway (Imler et al., 2003) in which IL-1R associated kinase (IRAK) 1 and 4 (Suzuki et al., 2002) are recruited to MyD88. IRAK-1 and -4 are then autophosphorylated and amplify the signal by binding to TNFR-associated factor (TRAF) 6. This in turn activates either the mitogen activated protein kinase (MAP3K) pathway leading to activation of activating protein 1 (AP-1) or the NFκB pathway (Smith et al., 2005). Both AP-1 and NFkB can bind to promoter regions of different responsive genes, including type I IFNs. TLR-7 and -9 seem to use this MyD88 dependent pathway to induce type I IFNs. Studies with MyD88-deficient mice have revealed additional adaptor molecules (Kawai et al., 1999). TIR-containing adaptor protein (TIRAP) was shown to be shared by TLR2 and -4 (Yamamoto et al., 2002), however, when using mice with both the MyD88- and TIRAP pathways disrupted, signaling via TLR3 and -4 was not completely disrupted as IFN-β still was expressed

(Oshiumi et al., 2003a; Oshiumi et al., 2003b). These findings resulted in the discovery of a third adaptor molecule called called TRIF (Toll/IL-1 receptor domain-containing adaptor inducing IFN-β) or TICAM-1 (TIR containing adaptor molecule) (Oshiumi et al., 2003a; Oshiumi et al., 2003b; Yamamoto et al., 2002). Furthermore, MyD88 independent TLR4 signaling requires yet another adaptor molecule called TRAM (TRIF-related adaptor molecule), also known as TICAM-2 (Fitzgerald et al., 2003a; Fitzgerald et al., 2003b). Hence, for the TLRs able to induce type I IFNs, at least two separate IFN inducing signaling cascades exist. First, the MyD88 dependent pathway utilized by TLR7 and -9 described above, and second, a MyD88 independent pathway used by TLR3 and -4, which at the end results in the phosphorylation and subsequent activation of IRF-3. The upstream events of IRF-3 phosphorylation require TRIF to associate with TRAF-6, which in turn leads to their association with tank binding kinase I (TBK1) and IKK-ε (Fitzgerald et al., 2003a; Fitzgerald et al., 2003b; Sato et al., 2003). This TRIF/TRAF-6/IKK-ε/TBK1 complex leads to the activation of IKK-ε and TBK1, which then allows the phosphorylation of the constitutively expressed IRF-3. The phosphorylated IRF-3 can now homodimerize, though lacks transciptional activity until binding the p300/CBP co-activators (Yang et al., 2002). Once active, the IRF-3 complex can bind the promoter region of the type I IFNs.

A hierarchy of temporal expression within the type I IFN gene family has been proposed (Juang et al., 1998; Marie et al., 1998). IFN-β and IFN-α4 are so called early responders whose transcription, in principle, is promoted by IRF-3 (Sato et al., 2000). IFN-non-α4s, the enhancers of which do not contain IRF-3 binding sites, are delayed responders and require activated IRF-7 for transcription (Doly et al., 1998; Lopez et al., 1998). IRF-7, unlike IRF-3, is not constitutively expressed, and its expression is induced by signals from the type I IFN receptor in a positive feedback loop (Sato et al., 1998).

Despite of their shared responsiveness to IRF-3, a further hierarchy was revealed between IFN-β and IFN-α4 in IFN-$\beta^{-/-}$ mice (Deonarain et al., 2000; Erlandsson et al., 1998). In primary embryonic fibroblasts, no IFN-α, including IFN-α4, was induced in primary embryonic fibroblasts upon infection with Sendai virus (SeV) (Erlandsson et al., 1998). In contrast, using immortalized fibroblasts from mice that were unable to signal via the type I IFN receptor, it was shown that IFN-α4 can be induced without previous expression of IFN (Marie et al., 1998).

1.2.4 Type I IFN receptor

The major ligand-binding chain of the human type I IFN receptor (IFN-Rα, or IFNAR-1) was cloned by Uzé et al in 1990. It is a type I transmembrane protein with a predicted molecular mass of 63kDa. The extracellular chain is 409 amino acids in length and contains 2 fibronectin type III repeats (Bazan, 1990), the N-terminal of these repeats probably having the ligand binding function (Chill et al., 2002). The structure classifies the receptor as a class II cytokine receptor (Mogensen et al., 1999). The cytoplasmic domain of IFNAR-1 is only 100 amino acids long and has no intrinsic enzymatic activity.

The second subunit was cloned in 1994 and was originally believed to have one soluble and one transmembrane form (Novick et al., 1994). A third form with an extended cytoplasmic domain was cloned in 1995 (Domanski et al., 1995; Domanski et al., 1996; Lutfalla et al., 1995) and was able to, in contrast to the previously cloned transmembrane part, fully support signalling in response to type I IFN. Today there are three known forms of the IFN-Rβ (or IFNAR-2) subunit: one soluble, 2a, and 2 transmembrane forms (long, 2c, and short, 2b) generated by alternative splicing of the same transcript (Prejean et al., 2000) (Fig. 1.2) What differentiates the long form from the short form is the cytoplasmic domain. The long form is the signalling competent one, and it has seven tyrosines on the cytoplasmic domain, several of wich are required for function (Nadeau et al., 1999), and also 6 acidic domains (Domanski et al., 1995).

The short form only has two cytoplasmic tyrosines (Novick et al., 1994) and its function is so far unknown, though it might act as a rheostat for modulating cellular responses to the type I IFNs. The soluble form may function as a natural antagonist of the type I IFNs.

Both IFNAR-1 and IFNAR-1 map to chromosome 21 in the human and to chromosome 16 in the mouse. Two strains of mice with a disrupted IFNAR-1 gene have been reported (Hwang et al., 1995; Muller et al., 1994). No mice deficient in the IFNAR-2 chain have been reported so far. The IFNAR-1 deficient mice are phenotypically normal with only slight perturbations in immune responses in comparison to wild-type mice, though they are unable to mount an efficient antiviral response against an array of different viruses.

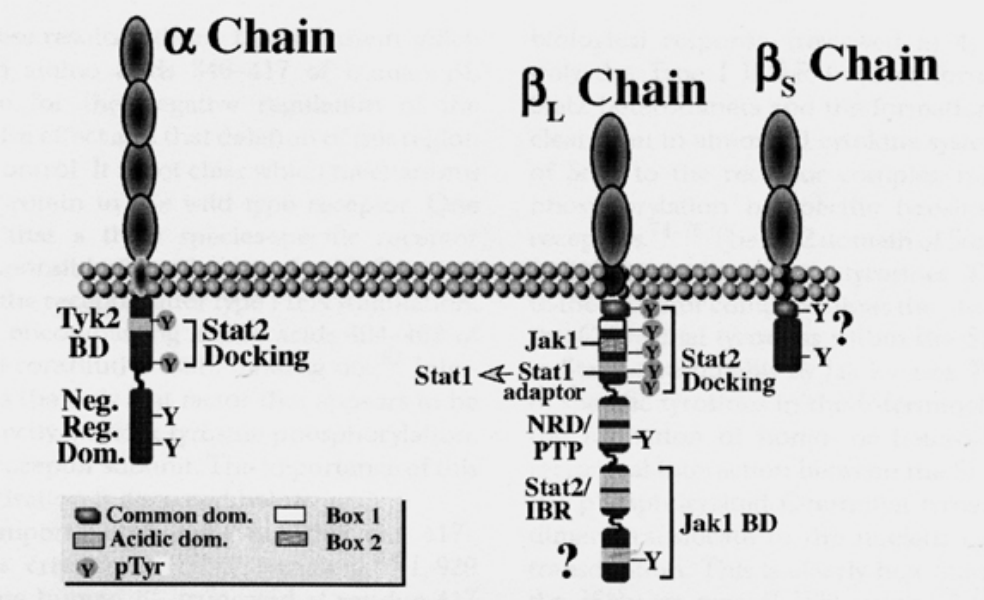


Fig. 1.2 Subunits of the type I IFN receptor. *Illustrated are the tyrosine phosphorylation sites (-Y), the Tyk2 and Jak1 binding domains, as well as the STAT2 docking sites. The α-chain is IFNAR-1, and the β_L- and and β_S-chains are IFNAR-2c and -2b respectively.*

1.2.5 Type I IFN signal transduction

One of the major pathways for the propagation of IFN signals is the Jak/STAT pathway. Jaks, or Janus kinases, are a family of cytoplasmic non-receptor tyrosine PTKs. Tyk2 and Jak1, two members of the Jak kinase family, are constitutively associated with the IFNAR-1 and IFNAR-2c subunits respectively (Colamonici et al., 1994; Colamonici et al., 1995; Domanski et al., 1997). They are activated via either trans- or autohosphorylation when IFNs bind to their receptor. The subunits are then rapidly phosphorylated on their cytoplasmic tyrosines (Platanias et al., 1999). The phosphorylated receptors now serve as docking sites for members of the STAT family of transcriptional co-activators, specifically STAT-1a and STAT-2 (Prejean et al., 2000). Both IFN-α and -β induce tyrosine phosphorylation of the receptor subunits, though only IFN-β, but not IFN-α, induces a tight association of the two chains. This qualitative difference in interaction of distinct type I subtypes with the receptor may account for the variation in biological responses to IFN subtypes (Abramovich et al., 1994; Croze et al., 1996; Lamken et al., 2004; Platanias et al., 1994; Platanias et al., 1996b).
A number of STAT proteins, including -1, -2, -3 and -5, can now be tyrosine phosphorylated (Platanias et al., 1999b). STAT-1 and 2 form a heterodimer in the form of interferon stimulated gene factor 3a (ISGF3a), which in turn associates with IRF-9 (p48) to form the mature ISGF3 transcriptional activator. The ISGF3 complex translocates to the nucleus and

binds to *cis*-acting DNA elements called interferon stimulated response elements (ISREs), which are palindromic sequences found in the promoter regions of IFN-responsive genes (Darnell, Jr., 1997; Darnell, Jr., 1998; Stark et al., 1998). A full transcriptional activation of ISREs by ISGF3 requires serine phosphorylation of STAT-1a by protein kinase Cδ (PKCδ) and PKCδ dependent activation of the p39 MAP kinase pathway (Uddin et al., 2002).
The above described signaling pathway today has the status of the classic IFN signaling pathway, and additional signaling pathways are proposed to exist. These might involve RNA-dependent protein kinase (PKR) (Kumar et al., 1997), mitogen-activated protein kinase (MAPK) (David et al., 1995; Goh et al., 1999) and phosphatidylinositol 3-kinase (Pfeffer et al., 1997). The final outcome of this signaling cascade is the transcription of interferon stimulated genes (ISGs), a few of which will be reviewed below.

1.2.6 Type I IFN induced genes and activities

Before the advent of DNA microarrays the ISGs were thought to consist of possibly 30-40 genes. Today, it is known that the ISGs are in the hundreds and include genes that are either repressed or stimulated by type I IFNs (de Veer et al., 2001; Der et al., 1998). Among these ISGs are several transcription factors that exert important effects on the immune system. One class are the IRFs, which have already briefly been mentioned, and consist of at least nine members (Theofilopoulos et al., 2004). They can all induce or be induced by type I IFNs. IRF-3 plays an important part in the initial expression of the type I IFNs. It is constitutively produced and activated by viral infections and TLR signaling. IRF-7 is induced by this first burst of type I IFN production being mediated by IRF-3, and is important for the propagation of the type I IFN positive feedback loop leading to a delayed, second burst of type I IFN production. Of the other IRFs, IRF-1 and -2 seem to have antagonistic roles, where IRF-1 is an activator of ISRE-containing genes and IRF-2 a suppressor. IRF-5 is also induced by viral infections and by type I IFNs but also by p53, and is thought to play a role in the expression of cell cycle regulatory and apoptotic genes, apart from involvement in anti-viral activity.
One of the first group of ISGs to be studied in virus infections were the myxovirus-resistance (Mx) proteins (Staeheli et al., 1987). The Mx proteins are GTPases that can cause redistribution of viral capsid proteins as a mechanism to inhibit viral replication.
One of the most extensively studied ISGs is the dsRNA-activated serine/threonine protein kinase (PKR) (Meurs et al., 1990). Activated PKR can negatively affect cell-regulatory pathways, primarily mRNA transcriptional and translational events.

Two other enzymes, 2',5'-oligoadenylate synthetase (OAS) and RNaseL, are responsible for mRNA degradation and are in a pathway seemingly activated by dsRNA (Zhou et al., 1993). They have important cell regulatory roles apart from being anti-viral.
As mentioned, these are only a few of hundreds of ISGs. In order to better view the complexity of the type I IFN response, and the resulting functions, the remainder of this section is divided into four subsections to explain the most important functional outcomes of type I IFN stimulation.

1.2.6.1 Antiproliferative functions

A few specific IFN-induced gene products have been linked to antiproliferative activity and it is known that both PKR and RNaseL are important mediators of the antiproliferative functions of IFNs. Overexpression of PKR leads to growth suppression and apoptosis in a number of cell types (Chong et al., 1992; Dever et al., 1993; Koromilas et al., 1992). Also overexpression of 2'-5' OAS reduces the growth rate in transfected cells (Chebath et al., 1987; Coccia et al., 1990). Furthermore, overexpression of RNaseL, activated by 2'-5' OAS, enhances the antiproliferative activity of IFN (Zhou et al., 1998) and it has also been shown that a dominant negative mutant of RNaseL inhibits the antiproliferative effects of type I IFN (Hassel et al., 1993).
Most genes linked with IFN-induced antiproliferative activity target specific components of the cell cycle control apparatus. These genes include c-myc, retinoblastoma gene product (pRB) and cyclin D3 (Kumar et al., 1992; Melamed et al., 1993; Resnitzky et al., 1992; Tiefenbrun et al., 1996). Type I IFNs also exert negative regulatory effects on the cell cycle by upregulating a number of cyclin-dependent kinase inhibitors (CKIs), e.g. $p21^{Cip1/Waf1}$ (Chin et al., 1996; Subramaniam et al., 1997; Subramaniam et al., 1998), which plays a critical role in the progression from G1 into S phase by binding to and decreasing the activity of Cdk2 (Gartel et al., 1996; Harper et al., 1993; Sangfelt et al., 1997). Other CKIs increased by type I IFNs are $p15^{Ink4b}$ that complexes with Cdk4 (Sangfelt et al., 1997), and $p27^{Kip1}$ which preferentially binds to cyclinE/Cdk2 complexes (Sangfelt et al., 1999). When levels of the CKIs are elevated, Cdk activity is reduced and the phosphorylation of the pRb, and the related pocket proteins p107 and p130, are repressed (Sangfelt et al., 1999). The phosphorlation of these three proteins normally releases E2F transcription factors permitting the transition from G1 to S phase. STAT-1 has also been shown to mediate antiproliferative IFN effects, as fibroblasts derived from STAT-1 deficient mouse embryos are not growth arrested by IFN-α (Bromberg et al., 1996).

IFNs are known to generally inhibit the growth of various untransformed and transformed cells, although cells in culture have varying degrees of sensitivity to the antiproliferative activity of type I IFNs. Daudi cells, for example, are very sensitive to IFN-α (Melamed et al., 1993) and are routinely used for studying the mechanisms of type I IFN induced G1 arrest (Subramaniam et al., 1998). Early-passage MEFs from $PKR^{-/-}$ mice on the other hand achieve saturation densities similar to that of WT cells, while MEFs grown for 5 passages or longer achieve higher saturation densities than WT cells (Stark et al., 1998). However, the doubling times of WT and $PKR^{-/-}$ MEFs are not different between early and late passages, and the difference in saturation densities might result from an increased resistance to apoptosis induced by growth-factor deprivation in the abscence of PKR.

IRF-1 and -2 are today viewed as the prototype IRFs involved in cell cycle control (Kirchhoff et al., 1993; Sato et al., 2001; Tanaka et al., 1993), and especially IRF-1 plays a key role, probably by its cell cycle checkpoint function. IRF-1 mRNA is markedly elevated in NIH3T3 cells that have undergone serum starvation and rapidly declines after adding serum (Harada et al., 1993a). Also, murine embryonic fibroblasts (MEFs) deficient in IRF-1 have impaired ability to to undergo DNA damage induced cell cycle arrest after exposure to genotoxic stress (Tanaka et al., 1996). Interestingly, anchorage-independent growth and tumor formation caused by injecting NIH3T3 cells overexpressing IRF-2 into nude mice is reverted by the concomitant expression of IRF-1 (Harada et al., 1993).

IRF-2 is thought to be involved in the activation of genes critical for cell proliferation, like certain histones functionally coupled to cell cycle progression (Vaughan et al., 1995), and restrained cell growth seems to depend on a balance between these two mutually antagonistic IRFs (Harada et al., 1998).

Other IRFs implied in cell growth control include IRF-4, which is exclusively expressed in lymphocytes, and is essential for the function and homeostasis of both mature B and T cells (Mittrucker et al., 1997). Similarly, IRFs-3, -8 and -9 have been shown to be able to function as tumor suppressors (Romeo et al., 2002). Recently, IRF-5 was shown to inhibit the growth of tumor cells both *in vitro* and *in vivo*, and growth inhibition was associated with a G2-M cell cycle arrest (Barnes et al., 2003).

Furthermore, there are potential IRF binding sites in many genes involved in growth control, some with antiproliferative activity and some necessary for growth (Romeo et al., 2002), thus explaining the pleiotropic effect observed on the cell cycle by type I IFNs.

1.2.6.2 Regulation of apoptosis

Apoptosis serves as a defense mechanism for the host cell to combat viral infection and type I IFNs have been shown to be essential mediators of apoptosis. Type I IFNs alone do not induce apoptosis, but only in association with dsRNA (Samuel, 2001). Primary MEFs undergo apoptosis when infected with certain viruses and this apoptosis can be inhibited by type I IFN antibodies. Also, apoptosis is blocked in IFNAR as well as in STAT-1 deficient MEFs (Tanaka et al., 1998).

Two ISGs, PKR and RNaseL, play key roles as effectors of apoptosis. Overexpression of WT PKR, but not catalytically inactive PKR, causes apoptosis in the prescence of dsRNA (Balachandran et al., 1998). In cells from mice with a disrupted RNaseL gene, apoptosis is suppressed following treatment with dsRNA or LPS (Zhou et al., 1997). A similar observation was made in PKR null cells, and the suppression of apoptosis was attributed to defects in IRF-1 activation and in Fas mRNA induction (Der et al., 1997).

Among the IRFs involved in apoptosis are IRF-1, -3, -5 and -8. Expression of the oncogene c-ras in combination with inhibitors of cell proliferation caused WT fibroblasts, but not IRF-$1^{-/-}$ fibroblasts, to undergo apoptosis (Tanaka et al., 1994). Also, virus activated IRF-3 has been shown to be a potent inducer of apoptosis, while myeloid cells derived from IRF-8 deficient mice are resistant to apoptosis induced by DNA damage (Barber, 2000). In accordance, IRF-5 has been show to induce multiple pro-apoptotic genes, like caspase-8, Bax and p21 (Barnes et al., 2003).

Recently, it was also shown that the tumor suppressor p53, essential for the induction of apoptosis in cancer, is induced by type I IFNs (Takaoka et al., 2003a). Type I IFNs do not themselves activate p53 but have a role in boosting p53 responses to stress signals. Furthermore, p53 is activated in virally infected cells and can evoke an apoptotic response. Induction of apoptosis by type I IFNs has been shown to involve FADD/caspase-8 signaling, activation of the caspase cascade, release of cytochrome c from mitochondria and DNA fragmentation, regardless of cell type (Chawla-Sarkar et al., 2003). Furthermore, type I IFN apoptotic induction occurs late, more than 48 h after treatment, implicating involvement of intermediate cellular effectors or genes activated by IFNs (Chawla-Sarkar et al., 2001). Finally, type I IFNs have recently been shown to be involved in inducing T cell apoptosis after *L.monocytogenes* infection (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004), and will be discussed later on.

1.2.6.3 Inflammation and immunomodulation

The type I IFNs also affect operational levels of the immune system, by being able to exert effects on most, if not all, cells of the immune system, thus playing a major part in inducing inflammatory responses. For example, type I IFNs are required for the development of a regular splenic architecture (Deonarain et al., 2003), are capable of positively or negatively affecting the generation, maturation and function of DCs (Bogdan et al., 2004), mediate cross-priming of $CD8^+$ T cells by APCs (Le Bon et al., 2003), enhance the cytotoxicity and cytokine production of NK cells (Nguyen et al., 2002), activate or deactivate macrophages, or sensitize the same cells to microbe-induced cell death (Stockinger et al., 2002). Apart from this, type I IFNs can also activate γ:δ T cells, prime Th1 and cytotoxic T cells and influence antibody production (Beignon et al., 2003).

Maybe one of the most important functions of type I IFNs are their ability to increase the expression of the peptide transporter TAP-1 and the upregulation of MHC-I (Cho et al., 2002) on DCs and other APCs, thereby promoting the development of $CD8^+$ T cell responses (Boehm et al., 1997). Mice with disruptions in the type I IFN signaling system, e.g. the IFNAR, STAT-1, PKR and IRF-1 proteins, fail to upregulate MHC-I on their cell surface when treated with appropriate stimuli (Stark et al., 1998). Moreover, the adaptive immune response is dependent on costimulatory surface molecules, e.g. CD40, CD80 and CD86, which are expressed on the surface of APCs. These molecules help to generate a sufficient response within T cells that are exposed to antigen on MHC-I or -II molecules. A recent study demonstrated that this upregulation can be induced by LPS and dsRNA in both TRIF-dependent and -independent ways (Hoebe et al., 2003), suggesting an involvment of IFN feed back loops.

Interestingly, type I IFNs seem to have a dual role in inflammation. Type I IFN is effectively used as a therapy against multiple sclerosis (MS) in humans. Accordingly, in the murine EAE model, IFN-$\beta^{-/-}$ mice show an increase in EAE progression and severity (Teige et al., 2003). Furthermore, IFN-$\beta^{-/-}$ mice also have more severe pulmonary inflammation in response to ovalbumin sensitization and challenge (Matheu et al., 2003). On the other hand, IFN-β deficient mice are resistant to toxic shock and IFN-β is essential for the toxic shock response *in vivo* (Karaghiosoff et al., 2003).

1.2.6.4 Infection (antiviral and antimicrobial activities)

The exclusive importance of the type I IFNs in anti-viral responses was first demonstrated in IFNAR deficient mice (van den Broek et al., 1995a). These mice displayed an extreme sensitivity to infection by viral pathogens. However, these experiments did not distinguish between the roles of IFN-α and -β in viral infections. A later study, in which an IFN-$\beta^{-/-}$ mouse was utilized, demonstrated that also these mice were highly susceptible to certain viral infections (Deonarain et al., 2000). Furthermore, this study also showed that viral induction of IFN-α and OAS was impaired in MEFs, setting the stage for IFN-β as the master type I IFN.
Among the several hundred genes that are transciptionally regulated by the type I IFNs, three families have been extensively studied with respect to their anti-viral activities. These genes encode PKR, OAS and the Mx proteins.
PKRs are cellular proteins with a regulatory domain that has dsRNA binding activity. Upon binding dsRNA, presumably a product of viral replication, PKR dimerizes, autophosphorylates and becomes activated. PKR can in turn phosphorylate eIF-2α, which is then inactivated leading to a general inhibition of protein synthesis. This includes the inhibition of viral protein expression and viral replication. Interestingly, $PKR^{-/-}$ mice show largely normal responses to viral infections, though vesicular stomatitis virus replicates to higher titers in the lungs of $PKR^{-/-}$ mice (Samuel, 2001).
OAS is, just like PKR, activated by dsRNA generated during viral infection. Active OAS can bind inactive monomeric RNaseL, inducing its dimerization and activation. Activated RNaseL cleaves both mRNA and rRNA in the cytosol of cells, leading to an inhibition of protein expression. The role of OAS in combatting viral infections is supported by the fact that $RNaseL^{-/-}$ mice are more susceptible to EMCV infection (Levy et al., 2001).
A third group of IFN-induced anti-viral proteins are the Mx GTPases. Not all of these have anti-viral activity but those that do, seem to be able to interfere with the trafficking and/or the transcriptional activity of viral ribonucleoprotein complexes (Weber et al., 2000), although this function has as yet not been fully validated.
Mice deficient in all three of the above described anti-viral proteins have been generated (Zhou et al., 1999), and are more sensitive to viral infection than wild type mice. Nevertheless, these mice were able to mount a measurable antiviral response and also show greater resistance to infection compared to mice lacking IFNAR or STAT-1, thus providing clear evidence for the existence of so far uncharacterized antiviral pathways induced by type I IFNs.

Compared to viral infections, the role of type I IFNs in the immune defense against other pathogens has been studied less. However, a few interactions and functional relationships between these pathogens and type I IFNs have been firmly established. Treating macrophages, or neutrophils, with either IFN-α or IFN-β enhanced their antimicrobial activity against *Mycobacterium avium*, *Toxoplasma gondii* and *Leishmania* spp. among other non-viral pathogens (Bogdan, 2000). Also, certain bacteria or protozoa induce production of type I IFNs in macrophages and fibroblasts *in vitro*, as well as *in vivo* (Diez et al., 1989; Havell, 1993), and recombinant mouse IFN-β protected mice against *L.monocytogenes* infection (Fujiki et al., 1988). Though, all these studies still had to elucidate the function of endogenously produced type I IFN, and how the type I IFNs exert their antimicrobial functions.

As *L.monocytogenes* is used as a model for bacterial induction of type I IFN in this work, a more detailed description about it, and the immune reactions against it, are given below.

Chlamydia is another family of bacteria that can induce type I IFNs via TLR-4 and the MyD88 dependent pathway. This in turn leads to production of IFN-γ, which is essential for the control of this pathogen *in vivo* (Bogdan et al., 2004).

Type I IFNs released in response to *M. tubercolosis* is required for the expression of IP-10, a known activator of NK cell and T cell recruitment (Lande et al., 2003), though $IFNAR^{-/-}$ show an almost unaltered resistance to *M. tubercolosis* (Bogdan et al., 2004).

Type I IFNs also seem to play a role in protozoan infections, Leishmaniasis being the best studied model to date. During the innate phase of response to *L. major* infection the expression of iNOS, the cytotoxic activity of NK cells, and the early production of IFN-γ all were dependent on enodgenous release of type I IFNs (Diefenbach et al., 1998). Another parasite which has been studied with respect to type I IFN protection against microbial infection is *Trypanosoma cruzi*. In this model, type I IFN mediated signaling is not required for the control of the parasite *in vivo*, as the $IFNAR^{-/-}$ mouse had intact IFN-γ production and was not more susceptible to infection even though it had an absent NK cell cytotoxic response during the early phase of infection (Une et al., 2003).

In summary, the exact mechanism by which type I IFNs acts in non-viral anti-microbial defense is still unclear, though a possibilty is that the secretion of type I IFNs from infected cells activate NK cells. These NK cells are then activated and produce IFN-γ, which in turn signals back to the infected cells, e.g. macrophages, and enhances their ability to kill the

ingested pathogen. Also, why type I IFNs are only of importance at certain stages of infection and not against all non-viral pathogens remains unclear.

1.3 *Listeria monocytogenes*

One of the microbial pathogens used in this work to study the biology of the type I IFNs is *L.monocytogenes*. *L.monocytogenes* is a gram-positive, facultative, highly motile rod that causes an uncommon but potentially serious type of food-borne infection, called listeriosis. Especially pregnant women, the elderly and other immunocompromised persons are susceptible to *L.monocytogenes*, while immunocompetent persons usually are able to rapidly clear infections.

L.monocytogenes is normally found in soil, water, the intestinal tracts of many animals but also on a variety of foods, especially industrially produced foods like soft cheeses and dairy products. One of the traits of *L.monocytogenes* that makes it difficult to control during food processing is that it has the ability to multiply at high salt concentrations and over a wide range of temperatures, including refrigerating temperatures (Bibb et al., 1990).

L.monocytogenes is an invasive pathogen that can enter phagocytic as well as non-phagocytic cells types, and the natural route of infection is through the gastrointestinal tract. The key features of its intracellular life cycle are illustrated in Fig. 1.3. The cycle starts with the adhesion of *L.monocytogenes* to the cell membrane of the host cell. The two main bacterial factors involved in this initial step are internalin A (InlA) and B (InlB). InlA binds specifically to E-cadherin expressed on epithelial cells (Gaillard et al., 1991) in humans, though has no binding activity to the mouse homologue of E-cadherin, due to a single amino acid exchange (Lecuit et al., 2001). After crossing the epithelial cell barrier, *L.monocytogenes* disseminate through the bloodstream to other organs, like the spleen and liver, where they are internalized by splenic and hepatic macrophages. *L.monocytogenes* enters hepatocytes through the expression of InlB which binds to the hepatocyte growth factor receptor (Shen et al., 2000).

After cellular invasion, *L.monocytogenes* escapes the phagosome by secreting listeriolysin O (LLO) (Bielecki et al., 1990), in synergy with phospholipase C B (plcB). Mobility of *L.monocytogenes* in the cytosol is enabled by expression of the actin-assembly inducing protein (ActA). This protein nucleates actin, thereby creating actin polymers that propel the bacteria through the cytoplasm and also into neighbouring cells (Domann et al., 1992; Kocks et al., 1992). Once in the neighbouring cell, *L.monocytogenes* again escapes the formed

vacuole through the disruption of the membrane, in a process dependent on LLO and plcB but also on phospholipase C A (plcA) (Camilli et al., 1993).

L.monocytogenes elicits a predominantly cell mediated immune response during infection in mammals and has been used by immunologists since the 1950s to study these types of immune responses. In fact, one of the best understood bacterial infection models to date is the murine listeriosis model (Kaufmann, 1993; Shen et al., 1998). One caveat using the murine listeriosis model is the fact that mice are relatively resistant to intestinal infection through the single amino-acid difference between the human and mouse E-cadherin (Lecuit et al., 2001). Therefore, most laboratory studies charaterizing the immune responses against *L.monocytogenes* use either intravenous or intraperitoneal infection routes, resulting in systemic infections.

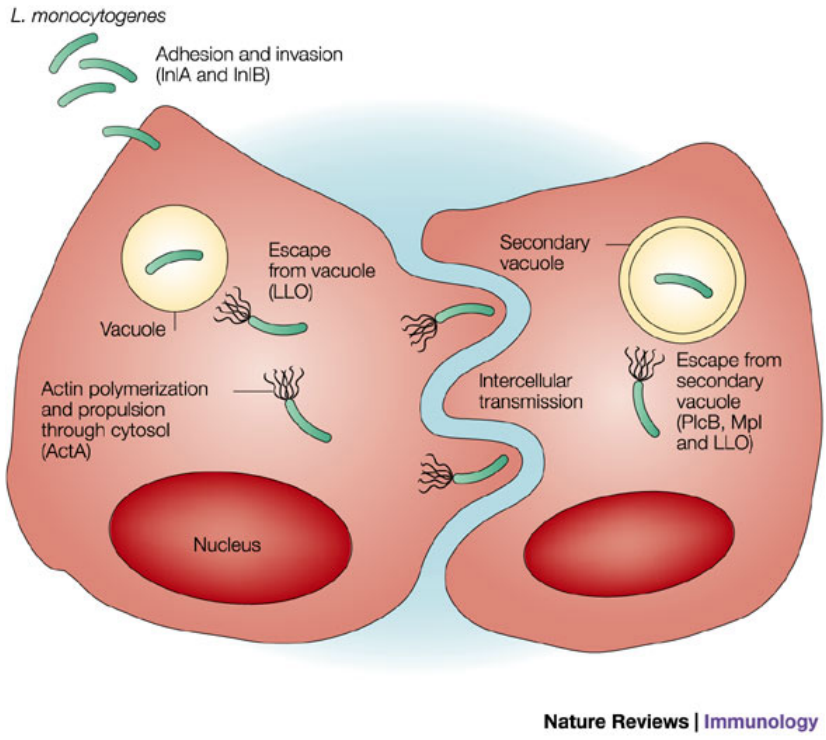


Fig. 1.3. The life cycle of L.monocytogenes. *The different stages and virulence factors involved are shown (Pamer, 2004).*

1.3.1 *L.monocytogenes* and innate immunity

Innate immune responses are rapidly triggered following infection with *L.monocytogenes* and are essential for host survival (Unanue, 1997). Neutrophils and macrophages are thought to be the principal mediators of *L.monocytogenes* killing on the cellular level. Depleting granulocytes in mice enhances their susceptibility to infection with *L.monocytogenes* (Conlan

et al., 1994; Czuprynski et al., 1994; Rogers et al., 1993), and experiments that block complement receptor 3 (Rosen et al., 1989) or CC-chemokine receptor 2 (Kurihara et al., 1997) have demonstrated the importance of monocyte recruitment for the clearance of *L.monocytogenes*. Recently, a novel DC type called TipDCs (TNF- and iNOS producing dendritic cells) (Serbina et al., 2003) were shown to play an essential role in the control of bacterial growth *in vivo*. TipDCs together with *L.monocytogenes* infected macrophages are the main producers of TNF-α and iNOS, two proteins essential for the defence against *L.monocytogenes* infection. Early resistance to infection is further attributed to IFN-γ production by NK-cells, which leads to activation of macrophages (Tripp et al., 1993). The complete activation of an innate immune response to *L.monocytogenes* is a multistep process, with sequential triggering of receptors and downstream signalling pathways. Toll-like receptors (TLRs) transmit signals in response to microbial molecules that activate innate immune defences (Takeda et al., 2004a+b). TLR2 and -5 have been implicated in the recognition of *L.monocytogenes* (Hayashi et al., 2001; Seki et al., 2002), although the importance of TLRs in triggering the innate immune response after *L.monocytogenes* infection is still unclear. *In vivo* data are so far lacking for TLR5 and the $TLR2^{-/-}$ mouse shows normal resistance to *L.monocytogenes* infection (Edelson et al., 2002; Serbina et al., 2003a).

Interestingly, adaptor proteins downstream of the TLRs show different phenotypes. $MyD88^{-/-}$ mice for example are very susceptible to *L.monocytogenes* infection (Edelson et al., 2002; Seki et al., 2002; Serbina et al., 2003a) as are $NFkB^{-/-}$ mice (Sha et al., 1995), while disturbing the type I IFN pathway increases resistance to infection, as was recently shown in mice lacking IRF-3 (O'Connell et al., 2004) or IFNAR (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). Type I IFNs therefore seem to impair the immune defence against *L.monocytogenes*, which is in contrast to their protective function in the innate immune response against viruses. Signalling elicited by type I IFNs after *L.monocytogenes* infection seems to decrease the clearance of bacteria by inducing apoptosis of T cells (Carrero et al., 2004; O'Connell et al., 2004) and the loss of TNF-producing cells (Auerbuch et al., 2004). Type I IFN were previously also shown to decrease the viability of *L.monocytogenes* infected macrophages (Stockinger et al., 2002).

In two recent studies it has been proposed that induction of type I IFN by intracellular bacteria is mediated by a TLR-, RIP2 and NOD2-independent but IRF-3 dependent pathway, that also

involves TBK-1 (O'Connell et al., 2005; Stockinger et al., 2004), though the complete signaling pathway is still not known.

1.3.2 *L.monocytogenes* and adaptive immunity

L.monocytogenes has been used even more extensively to characterize T-cell mediated immune responses. Mackaness early on demonstrated the cellular basis for protective immunity using *L.monocytogenes* (Mackaness, 1962), and subsequent studies showed that T cells mediate the clearance of *L.monocytogenes* (McGregor et al., 1970).
Humoral immunity only provides a small contribution to the protective immunity, even though antibodies specific for LLO ameliorates infection (Edelson et al., 2001). The γ:δ T cells seem to play a role in controlling inflammatory responses to *L.monocytogenes* infection (Egan et al., 2000), but α:β T cells are the major players in clearing bacterial infection and in providing long-term protection. $CD8^+$ T cells contribute more to the long-term protection than $CD4^+$ T cells do (Ladel et al., 1994), and these $CD8^+$ T cells can be divided into two populations. The first is restricted by classical MHC class I molecules (MHC class Ia), and the second is restricted by the non-classical MHC class I molecule H2-M3. These two populations respond to bacterial infection with distinct kinetics and provide distinct contributions to immune memory responses (Kerksiek et al., 1999; Seaman et al., 2000).

1.4 Virus

A vast amount of data on induction and effect of type I IFNs are derived from infection experiments using viruses. These microorganisms are small cellular parasites unable to reproduce by themselves. Once a virus infects a cell it can direct the cellular machinery to produce viral material and reproduce more viruses.
Viruses are classified according to the pathway they use to form mRNA and by the composition of their genomes. The simplest distinction is the one between DNA- and RNA viruses.
The DNA viruses comprise Class I and Class II viruses. The class I viruses, e.g. adenoviruses and Simian virus 40 (SV40), contain a double stranded DNA (dsDNA), which can be directly used to produce mRNA. The class II viruses, e.g. parvoviruses, have either a positive (+) or negative (-) single stranded DNA (ssDNA). The positive strand can be directly copied into mRNA, while the negative strand first needs to copy itself into dsDNA before being transcriptionally competent.

The RNA viruses are subdivided into four classes. Class III viruses, or reoviruses, contain dsRNA, and can directly produce mRNA. Class IV viruses, and class V viruses contain a (+) and a (–)ssRNA, respectively. The (+)ssRNA class IV virus needs to copy iteself into a (–)ssRNA before being able to produce mRNA, while the class V viruses have the ability to do so directly. The final class of viruses are the class VI or retroviruses. They contain a (+)ssRNA that directs the formation of a DNA molecule which integrates into the host genome and ultimately acts as a template for making viral mRNA.

As previously mentioned two TLRs are important in monitoring viral infections. TLR-3, which binds dsRNA, and TLR-7 (in mice, TLR-8 in humans), which recognizes ssRNA. Both these TLRs can induce type I IFNs, and TLR-7 is especially important as it is highly expressed on natural IFN producing pDCs (Lund et al., 2004).

Apart from inducing type I IFNs after infection, viruses also induce other proinflammarory cytokines, e.g. TNF-α, IL-1 and IL-6, in innate immune cells as well as certain nonimmune cells. These cytokines can be detected early in most viral infections, though the amounts produced may vary according to the cell tropism of the virus together with the ability to infect macrophages and DCs. The importance of NK cells in the early defense against a variety of viral infections has firmly been established (Biron et al., 1999). The NK cell mediated defense is dependent on the production of IFN-γ and downstream effects of this cytokine. NK cells also have the potential to promote resistance to infection as a result of TNF-α production. TNF-α in turn can promote the migration of DCs from peripheral compartments to carry antigen to and present it in lymphoid organs (Roake et al., 1995a; Roake et al., 1995b). Furthermore, NK cells can also recognize infected cells in which the virus has shut off class I MHC expression. In the normal state the presence of MHC-I on cells keeps NK cells in an inhibited state.

The adaptive immune response to viruses can be mediated by antibodies, $CD8^+$ cytotoxic T cells, as well as $CD4^+$ T cells. The antibodies are produced and bind to the viruses only during their extracellular life-stage, which can prevent viruses from binding to and entering host cells.

The $CD8^+$ cytotoxic T cells (CTLs) are important in eliminating viruses residing within cells. They recognize viral antigens presented on MHC-I molecules on any nucleated cell. For full differentiation, the CTLs require cytokines produced by $CD4^+$ helper T cells or costimulators expressed on infected cells. However, often IFN-γ is more important than the cytotoxic response and explains why $CD4^+$ T cells can play an important role in clearing virus infection.

1.4.1 Sendai virus

Sendai virus (SeV) has been used in this work as a stimulator of type I IFNs in murine fibroblasts. SeV is an enveloped linear (–)ssRNA respirovirus belonging to the family *paramyxoviridae*, and is a respiratory pathogen of laboratory mice. The SeV genome contains the nucleocapsid (N) gene, phospho (P) gene, matrix (M) gene, fusion (F) gene, hemagglutinin-neuraminidase (HA), and large (L) gene. Gene expression is usually monocistronic, generating a single mRNA which primarily directs a single translation product (Kato et al., 2001).

Even though SeV now is known to antagonize type I IFN signalling (Goodbourn et al., 2000) through its V or C proteins encoded by the P gene, it has long been known for its abilty to strongly induce type I IFN production upon infection and therefore remains a useful tool for studying type I IFN induction in the context of virus infections.

1.5 Aims of this work

The overall aim of this work was to extend the knowledge of the role of IFN-β during inflammation and infection. Using a previously generated IFN-β deficient mouse (Erlandsson et al., 1998), both *in vitro* and *in vivo* experiments were conducted.

Special interest was directed towards settling the issue of the temporal hierarchy of type I IFNs in fibroblasts after virus infection *in vitro*, as previously published studies had generated conflicting data on this subject (Erlandsson et al., 1998; Marie et al., 1998). This should be accomplished by comparing primary murine embryonic and adult fibroblasts from both wild type IFN-$\beta^{-/-}$, and IFNAR$^{-/-}$ mice. Furthermore, immortalization of these primary MEFs and MAFs was a prerequisite for settling the issue.

In addition, these cell lines should be used in cell growth studies to elucidate whether MEFs and MAFs lacking IFN-β demonstrate normal cell growth or not, as type I IFNs are known to have anti-proliferative effects.

Type I IFNs have also been shown to play a role in bridging the innate and adaptive immune systems. Therefore, it was interesting to clarify whether IFN-β deficient antigen presenting cells have the same ability as WT cells to stimulate an adaptive immune response. Macrophages and dendritic cells derived from bone marrow should therefore be stimulated with pathogen or pathogen specific molecules. Thereafter, their ability to stimulate T cells *in vitro* should be characterized.

Another part of this work was to study the importance of IFN-β in bacterial infection. Using the model pathogen *L. monocytogenes,* the ability of WT and IFN-$\beta^{-/-}$ mice to cope with such an infection should be compared. Furthermore, *in vivo* T cell responses in infected WT and IFN-$\beta^{-/-}$ mice should be studied.

Finally, IFN-β has been a dichotomy with regards to inflammation. For example, IFN-β is an essential effector of LPS induced lethality in a toxic shock model, though on the other hand acts to ameliorate symptoms in MS and the murine EAE MS-model. Further inflammatory models should therefore be sought for broadening our understanding of IFN-β and its role in inflammatory processes.

2 Materials and Methods

2.1 Mice

Female BALB/cOlaHsd (H-2^d) and C57Bl/6 (H-2^b) mice were purchased from Harlan Winkelman (Borchen, Germany) and used at age 8-12 weeks for all experiments. IFN$\beta^{-/-}$ mice (Erlandsson et al., 1998) were backcrossed onto the BALB/c background for 15 generations, and IFNAR$^{-/-}$ mice (Muller et al., 1994) were backcrossed onto C57Bl/6 for 10 generations.

2.2 Bacterial strains and culture conditions

L.monocytogenes strain EGDe (Leimeister-Wachter et al., 1989) was grown in Brain Heart Infusion (BHI) broth or on BHI-agar plates (Difco, Detroit, MI) at 37°C over night. The next day suspensions were diluted 1:50 and incubated for an additional 3-4 h at 37°C until reaching log-phase (OD_{600} ca 0,8). Bacteria were then washed and resuspended in sterile PBS.

2.2.1 Estimation of bacterial numbers

Bacteria concentration was determined after washing. Bacteria were diluted 1:4 in sterile PBS and OD was measured at 600 nm. The OD_{600} value was then compared to an established growth curve of *L.monocytogenes* (see below; Hense, PhD thesis, TU Braunschweig, 1999). The estimated concentration of colony forming units (CFU) of Listeria was always confirmed by plating aliquots of inoculums on BHI plates.

OD_{600}	CFU/ml
0.005	$4.2x10^6$
0.007	$8.56\ x10^6$
0.016	$1.34\ x10^7$
0.024	$2.17\ x10^7$
0.042	$5.53\ x10^7$
0.067	$1.1\ x10^8$
0.099	$1.68\ x10^8$
0.181	$5.44\ x10^8$
0.427	$1.53\ x10^9$
0.583	$4.57\ x10^9$
0.706	$1.57\ x10^{10}$
0.775	$3.3\ x10^{10}$
0.841	$5.64\ x10^{10}$
0.862	$1.52\ x10^{11}$

2.3 Virus strains

Sendai virus (SeV; Murine Parainfluenza Virus 1), 4800 hemagglutinating units (HAU)/ml, was originally obtained from the American Type Culture Collection (ATCC VR-105). Mouse Encephalomyelitis Virus (EMCV), $2x10^8$ plaque forming units (PFU)/ml, was also originally purchased at ATCC (VR-9955). Both virus strains were provided by Dr. R. Zawatzky (DKFZ, Heidelberg, Germany).

2.4 Cell lines and culture conditions

L-M(TK^-) (ATCC CCL 1.3), primary embryonic/adult fibroblasts and immortalized embryonic/adult fibroblasts were cultured in IMDM (Gibco BRL, Eggenstein, Germany) supplemented with 10% heat inactivated FCS (Integro, Zaandam, The Netherlands), 100 µg/ml penicillin/streptamycin (Biochrom AG, Berlin, Germany) and 250 µM β-mercaptoethanol (Serva, Heidelberg, Germany). Cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere.

Embryonic fibroblasts from wild-type, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice were prepared from day 14 embryos as previously described (Erlandsson et al., 1998).

Adult fibroblasts from wild-type and IFN-$\beta^{-/-}$ mice were prepared from ears of 8-10 week old female mice. Briefly, part of the ear was cut off after mice were sacrificed. Ears were then sterilized before being cut into small pieces that were distributed in 24-well cell culture plates. Complete IMDM was added and medium was changed every third day. After 2-3 weeks wells were confluent and cells were further propagated or frozen for later use.

Immortalization of fibroblasts with the SV40 large T antigen has been described elsewhere (Jat et al., 1989).

Cell numbers were always determined by trypan blue exclusion in a 0.1mm/0.025 mm² Neubauer cell chamber (Brand, Wertheim, Germany).

2.5 Virus infections

To study the induction of IFNs, primary fibroblasts in passage 4 were infected with SeV, while immortalized fibroblasts were kept in culture for at least 20 passages before stimulation with SeV. Confluent monolayers of fibroblasts were infected in serum-free IMDM using 24 HA units/ml SeV for 1 h. Cells were then washed in and incubated with complete IMDM for another 1, 5 or 23 h.

2.6 Type I IFN-bioassay

Supernatants of infected cells were collected either immediately after 1 h of virus infection and subsequent change of medium, and after additional culture for 1, 5 or 24 h. The total amount of secreted type I IFN was then determined by a modified standard bioassay protocol (Vogel et al., 2003). Briefly, L-M(TK$^-$) fibroblasts were added to 2-fold serial dilutions of supernatants from control or virus infected fibroblasts in 96-well microtiter plates. After 24 h, the medium was aspirated and EMCV at a PFU of $2x10^3$ in complete IMDM was added to the wells. After 18-22 h, when all virus control wells showed 100% cytotoxicity, medium was aspirated, cells were washed in PBS and then fixed with 5% formalin. After discarding the fixative, wells were stained with 0.05% crystal violet in 20% ethanol, and plates were scored by comparison to a recombinant murine IFN-β standard (R&D Systems, Wiesbaden, Germany)

2.7 Preparation of RNA

2.7.1 RNA extraction

Total RNA from cells and cell cultures was prepared with the RNeasy MiniKit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Briefly, cells were washed 2x in PBS, then disrupted in buffer RTL supplemented with β-mercaptoethanol. Cell culture wells were scraped with rubber policemen and cell lysates collected in Eppendorf tubes. The samples were then homogenized by passing the lysates through a 20-gauge needle fitted to a 1 ml syringe. Subsequently, standard protocol was followed and the optional on-column DNase digestion step with the RNase-free DNase set (Qiagen, Hilden, Germany) was performed.

2.7.2 Determination of RNA concentration

RNA concentration of samples was determined by measuring optical extinction at 260/280 nm with a BioPhotometer (Eppendorf, Hamburg, Germany). For subsequent cDNA preparation 2 μg of RNA was used.

2.7.3 Preparation of cDNA

cDNA was prepared using 2 μg of RNA. Possible DNA contamination in total RNA was eliminated by incubation with DNaseI (Amersham Pharmacia Biotech, Freiburg, Germany).

Reaction: 2 µg RNA
4 µl RT-buffer 5x/First strand buffer (Invitrogen, Karlsruhe, Germany)
1 µl DnaseI
(total reaction volume = 20 µl)

Samples were then incubated at 37°C for 15 min and 65°C for 10 min, and were thereafter divided into two equal parts and incubated with oligo-d(T) according to the following protocol:

10 µl RNA (mix from above)
1 µl oligo-d(T)
3.5 µl H_2O

Samples were incubated at 70°C for 10 mins then put on ice. After cooling the master reaction mixture was added.

Master mix/sample: 2 µl 5x First strand buffer
2 µl 0.1 M DTT
1 µl dNTP, 10 mM (stock 100 mM)
0.5 µl RNasine (40 U/µl) (Promega, Madison, WI)

Master mix and samples were preheated for 2 min at 42°C then combined. Finally, 1µl Superscript II $RNaseH^-$ (200 U/µl; Invitrogen, Karlsruhe, Germany) was added to half of the samples. Samples were incubated for 60 min at 42°C and thereafter for 10 min at 95°C. 80 ml depc H_2O was added and samples stored at -80°C.

2.8 Virus load

Virus loads in infected MEFs and MAFs were investigated by Dr. Stefan Lienenklaus. Generated mRNA was used with random primers. (Continued)

RT-reaction/sample: 0.1 µl Random primers (300 ng)

1 µg RNA

1 µl dNTPs

5.9 µl H_2O

Samples were incubated for 5 min at 65°C, reverse transcription continued by adding:

4 µl 5x First strand buffer

2 µl DTT

0.5 µl RNasine

0.5 µl H_2O

1 µl Superscript II

Samples were then incubated for 60 min at 42°C and 15 min at 70°C. 30 µl H_2O was finally added.

2.9 Polymerase Chain Reaction (PCR)

2.9.1 Oligonucleotides

RT-PCRs were performed with the following primers:

GAP-DH (s) 5'-ACCACAGTCCATGCCATCAC-3'
(as) 5'-TCCACCACCCTGTTGCTGTA-3'

IRF-7 (s) 5'-CAGCGAGTGCTGTTTGGAGAC-3'
(as) 5'-AAGTTCGTACACCTTATGCGG-3'

IFN-β (s) 5'-CATCAACTATAAGCAGCTCCA-3'
(as) 5'-TTCAAGTGGAGAGCAGTTGAG-3'

IFN-α4 (s) 5'-GTGCTTTCCTCATGATCC-3'
(as)5'-GGTTGAGGAAGAGAGGG-3'

IFN-non-α4 (s) 5'-ARSYTGTSTGATGCARCAGGT-3'
(as) 5'-GGWACACAGTGATCCTGTGG-3'

SeVNP (s) 5'-CTGCAGACCCTGACACACTC-3'
(as) 5'-CCT CAA CTG TCT CCC CAG TG-3'

2.9.2 RT-PCR

RT-PCRs were run on Primus96plus thermocyclers (MWG Biotech, Ebersberg, Germany). cDNA amounts and reaction efficiency were controlled with the GAP-DH housekeeping gene.

Reaction mix:

0.5 ul sense primer (10 pmol/µl)
0.5 ul antisense primer (10 pmol/µl)
2 ul 10x PCR buffer
2 ul NucMix (2mM)
14 ul depc Water
0.1 ul AmpliTaq Gold
19 ul in each tube and 1 ul sample cDNA

PCR programs:

IRF-7

95°C	10 min
30 cycles:	
94°C	20 sec
53.3°C	40 sec
72°C	1 min
Final extension :	
72°C	5 min

IFN-β

95°C	10 mins
30 cycles:	
94°C	20 sec
62°C	60 sec
72°C	30 sec
Final extension:	
72°C	5 min

IFN-α4

95°C	10 mins
34 cycles:	
94°C	20 sec
57°C	60 sec
72°C	30 sec
Final extension:	
72°C	5 min

(Continued)

IFN-non-α4

95°C	10 mins
36 cycles:	
94°C	20 sec
57°C	60 sec
72°C	30 sec
Final extension:	
72°C	5 min

GAP-DH

95°C	10 mins
26 cycles:	
94°C	20 sec
60°C	60 sec
72°C	30 sec
Final extension:	
72°C	5 min

SeVNP

72°C	10 mins
22 cycles:	
72°C	30 sec
58°C	30 sec
95°C	30 sec
Final extension:	
72°C	5 min

2.9.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate linearized, double-stranded DNA. 2% agarose (Appligene, Heidelberg, Germany) was used in TBE buffer, and gels were run at 120 V. Gels were stained with ethidium bromide, visualized with UV light and documented.

2.10 Immunological methods

2.10.1 Flow cytometry

Flow cytometry can determine the size and granularity of cells. When cells are stained with appropriate antibodies conjugated to fluorescent dyes, this method allows sensitive detection of cell surface molecules.

Cells for flow cytometric analysis were obtained from cell cultures or from homogenized organs. In general, single cell suspensions were prepared in FACS-buffer at a density of 1-2x10^5 cells/well in 96-well plates. Cells were then treated with anti-mouse FcR antibodies (Abs), to block Fc-receptors, for 10 min on ice. After washing, cells were stained with Abs

from the list below for 10 min on ice. Then cells were washed twice after the first staining, and biotinylated Abs were then counterstained with conjugated APC- or PE-streptavidin (SA) for 10 min on ice. Cells were again washed twice and resuspended on FACS-buffer. Just before flow cytometric analysis 1 μg/ml propidium iodide (PI; Sigma Aldrich, Taufkirchen, Germany) was added to reveal dead cells. For analysis a FACSCalibur (Becton Dickinson, Heidelberg, Germany) was used and data were analysed CellQuest Pro or WinMDI software.

FACS-buffer: PBS supplemented with 2% FCS, 2mM EDTA and 0.1% azide

2.10.2 Magnetic cell sorting

Magnetic cell sorting (MACS) was developed to separate cells with antibody- or streptavidin conjugated to magnetic microbeads. Cells labelled with magnetic beads are then passed through a separation column which is placed in a magnetic field. Magnetically labelled cells are retained in the column and unwanted unstained cells run through the column. The column is then removed from the magnet and the positively selected cell fraction can be eluted. This method was used in this work to obtain highly pure peptide specific CD8 T cells.

MACS-buffer: PBS supplemented with 0.5% BSA, pH 7.2

2.10.3 Antibodies

hamster-anti-mouse CD80 PE (B7.1; Pharmingen, Heidelberg, Germany)
rat-anti-mouse CD86 biotin (B7.2; Pharmingen)
rat-anti-mouse CD8 PE (Pharmingen)
rat-anti-mouse CD8 APC (Caltag, Burlingame, CA)
rat-anti-mouse CD62L FITC (Pharmingen)
rat-anti-mouse ERTR-9 biotin (BMA Biomedicals, Augst, Switzerland)
rat-anti-mouse MOMA-1 biotin (BMA Biomedicals)
rat-anti-mouse Dx5 FITC (Pharmingen)
rat-anti-mouse B220 FITC (Pharmingen)
hamster-anti-mouse CD3 biotin (Pharmingen)
*rat-anti-mouse CD11b FITC (anti-Mac-1, clone: M1/70.15.11.5, ATCC)
*rat-anti-mouse CD11c biotin (N418, clone: CHB 229, ATCC)
*rat-anti-mouse F4/80 (F4/80, clone: HB 198, ATCC)
*rat-anti-mouse CD40 FITC (clone FGK 45.5)

*rat-anti-mouse Gr-1 FITC (clone RB6-8C5)

*rat-anti-mouse CD54 FITC (ICAM-1, clone YN1/1-7)

*rat-anti-mouse MHC class II (anti I-$A^{k,d,b}$, clone 17/227)

*rat-anti-mouse MHC class I (anti H-$2D^{d}$, clone 34-4-20S)

* marked antibodies were isolated from culture supernatants and conjugated according to standard protocols.

2.11 Proliferation studies

2.11.1 Growth curves

Immortalized MEFs were grown until confluency in 75 cm^2 cell culture flasks, before being trypsinized and washed. Cell numbers were then determined by trypan blue exclusion. Fibroblasts were seeded out in 24- or 6-well cell culture plates in the amounts specified for each experiment. Total culture medium volume was 500 ul for 24-wells and 1 ml for 6-wells. Cells were then incubated at 37°C until trypsinized and counted by trypan blue exclusion. Primary MAFs were grown until passage 4 before being collected and treated like stated for the immortalized MEFs. MAFs were seeded out in 6-well plates, 10,000 cells/well in a total volume of 1 ml. rmIFN-β (R&D Systems, Wiesbaden, Germany) was added to both WT and IFN-$\beta^{-/-}$ MAFs at a concentration of 100 U/ml. MAFs were incubated at 37°C until trypsinized and counted by trypan blue exclusion.

2.11.2 CFSE labeling

Primary MAFs were treated like above. After determining cell number, MAFs were stained with the Vybrant CFDA SE (CFSE) cell tracer kit (Molecular Probes/Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions. 10,000 cells/well were then seeded in 6-well cell culture plates at a total volume of 1 ml. Cells were collected at different time points and fluorescence was measured on a FACSCalibur (Becton Dickinson, Heidelberg, Germany). FACS data was analysed with the provided CellQuest Pro software.

2.11.3 Cell cycle analysis

Primary MAFs were treated as previously described. After determining the cell number, MAFs were seeded out in 75 cm² cell culture flasks at $1x10^5$ MAFs/flask. MAFs were then collected at different time points after plating, washed in PBS and fixed in 500 µl ice cold 80% methanol. Samples were stored at 4°C until further use. When samples from all time

points had been collected, MAFs were washed with 0,1% saponin (Sigma Aldrich, Taufkirchen, Germany). Pellets were resuspended in 0,1% saponin supplemented with 20 mg/ml propidium iodide (PI) and 1 mg/ml RNase R-6000 (Sigma Aldrich, Taufkirchen, Germany) and incubated at 37°C for 30 minutes. Cell cycle stages were measured with a FACSCalibur (Becton Dickinson, Heidelberg, Germany) and analysed with ModFit LT 2.0 and WinMDI 2.8 software.

2.12 Isolation and stimulation of bone marrow derived APCs

2.12.1 Isolation of bone marrow derived APCs

Bone marrow macrophages and dendritic cells were isolated from 8-12 week old WT and IFN-β deficient BALB/c mice. Briefly, the femurs and tibias of mice were collected and flushed out with complete IMDM. Cells were collected in 15 ml Falcon tubes and centrifuged. Pellets were resuspended in ACT lysis buffer and pooled. Erythrocyte lysis was stopped after 5 min with complete IMDM and cell suspensions were again centrifuged. Pellet was resuspended in IMDM and distributed into 100 mm Petri dishes (Sarstedt, Nümbrecht, Germany) in a total volume of 10 ml IMDM/dish. Cells were incubated at 37°C for 2 h. Thereafter, non-adherent cells in supernatants were collected in 50 ml Falcon tubes. Plates were gently washed in IMDM, combined with supernatants obtained before and centrifuged. Plates with adherent cells were to be differentiated into bone marrow macrophages, and were therefore given 15 ml complete IMDM supplemented with 20% conditioned medium containing M-CSF derived from supernatants of the L929-580 cell line. At day 5 after plating, supernatants were discarded, plates gently washed and fresh IMDM supplemented with 20% conditioned medium was added. After an additional 2 days of culture, plates were washed and adherent macrophages were collected by gentle scraping.

The non-adherent cell were to be differentiated into bone marrow derived dendritic cells. To this end, cells were resuspended in complete IMDM containing 0.5 ng/ml GM-CSF (Sigma-Aldrich, Taufkirchen, Germany) and concentrated conditioned medium containing IL-4 from supernatants of X63/0 BHG Neo-mIL4 cells (working solution 1:400 as determined by testing supernatant on IL4 dependent CT4S cells) and seeded in 100 mm Petri dishes with a total volume of 15 ml supplemented IMDM. 5 ml of the supernatant was carefully discarded on day 3, 5 and 7 after seeding, and 5 ml freshly supplemented IMDM was added.

BMMϕ were collected on day 7 and BMDC were collected on day 8 after initial plating. Purity of cells was analysed by flow cytometry. BMMϕ were routinely >92% positive for

CD11b (Mac-1) and F4/80. BMDCs were >90% positive for CD11c (N418) and Gr-1 negative.

2.12.2 Stimulation of bone marrow derived APCs

Bone marrow APCs generated as described above were plated out at $1x10^6$ cells /well in 6-well cell culture plates. BMMϕ were allowed to adhere over night before stimuli was added, while BMDC were collected and immediately stimulated after analysis.
APCs were stimulated for 24 h with either 0.1μg/ml LPS (Fluka, Buchs, Switzerland), 30 mg/ml poly I:C (Amersham Biosciences, Freiburg, Germany), 1 mg/ml LTA (InvivoGen, San Diego, CA) or SeV (described in section 1.5) in a total volume of 1.5 ml IMDM/well.
After 24 h APCs were collected and either analysed in flow cytometer for their cell surface markers or used in a T cell stimulation assay to test their antigen presenting capabilities (see 1.12).
Antibodies used for flow cytometry were CD40, CD80 (B7.1), CD86 (B7.2), CD54 (ICAM-1), MHC class I and MHC class II.

2.13 Antigen presentation assay

Bone marrow derived APCs that had been stimulated with different PAMPs (section 1.11.2) were collected and counted by trypan blue exclusion. BMMϕ were then seeded in 96-well cell culture U-bottom plates at a density of $5x10^3$ cells/well, and BMDCs at a density of $1x10^3$ cells/well. Peptide comprising the sequence AA518-526 of hemagglutinin from Influenza virus PR8 was added at different concentrations. Finally, $1x10^4$ MACS-purified $CD8^+$ T cells from CL4 transgenic mice were added to the wells and plates were incubated for 3 days. For the final 16 h cultures were pulsed with 1 μCi [^{3}H]thymidine (Amersham Biosciences, Freiburg, Germany). T cell proliferation was measured after harvesting as [^{3}H]thymidine uptake on a 1450 MicroBeta liquid scintillation counter (Wallac, Turku, Finland).

2.14 Infection of mice with *L.monocytogenes*

2.14.1 Intravenous infection

Female mice were intravenously (i.v.) infected with approximately $2x10^3$ CFU ($0.4xLD_{50}$) of *L.monocytogenes*. Graded amounts of recombinant murine IFN-β (R&D Systems, Wiesbaden, Germany) was in some experiments injected 1 h prior to the Listeria.

CFU of injected Listeria was confirmed as previously stated. Depending on experiment, mice were sacrificed with CO_2 at different time points post infection.

2.14.2 Determination of number of viable bacteria in mouse tissues

Mice were sacrificed at day 2, 3, 4 and 5 post infection. Spleens and livers were removed and were then homogenized in 1 ml PBS supplemented with 0.2% NP-40. Serial dilutions of homogenates were plated on BHI agar plates in duplicate, and CFU per organ were estimated after over night incubation at 37°C.

2.14.3 Analysis of type I IFN expression in splenic cell populations

Female mice were intravenously (i.v.) infected with approximately $5x10^5$CFU of *L.monocytogenes*. Spleens were the collected either immediately or after 4 and 24 h p.i. After homogenisation of the spleens and lysis of erythrocytes, cells were stained for various cell type specific antibodies and sorted on a FACS Vantage DiVa. RNA isolation and RT-PCRs were performed as previously described. Data was kindly contributed by Dr. Jadwiga Jablonska.

2.15 *In vivo* cytotoxicity assay

WT and IFN-$\beta^{-/-}$ BALB/c female mice aged 8-12 weeks were infected with *L.monocytogenes* as described before. Control mice were intravenously injected with PBS. At days 4, 5 and 6 post infection an *in vivo* cytotoxicity assay was performed. On each of these days the procedure below was followed: naive WT and IFN-$\beta^{-/-}$ mice (1 mouse per 3 recipient mice) were sacrificed and spleens were collected. Spleens were minced through a 100 μm nylon mesh and pooled in 15 ml Falcon tubes. Cells were centrifuged for 10 min at 1,000 rpm, supernatant aspirated and erytrocytes lysed in ACT lysis buffer for 5 min at room temperature. Tube was filled with complete IMDM and cells were centrifuged as previuosly stated. Supernatant was aspirated and cells resuspended in 5 ml complete IMDM and filtered through a 30 μm Cell trics mesh (Partec, Münster, Germany) into a new 15 ml Falcon tube. Filters were washed once with 5 ml complete IMDM, and viable cells counted using trypan blue exclusion. Cell yield was approximately $1\text{-}2x10^7$ cells per donor mouse.
Cells were diluted in 5 ml complete IMDM per donor mouse, and the cell suspension divided into two equal volumes. One half served as control cells and an equal volume of complete IMDM was added to the cells. The other half was sensitized with $LLO_{91\text{-}99}$ peptide at an end

concentration of 1 μg/ml. Peptide was added to cells in an equal volume of complete IMDM. Both control and LLO_{91-99} sensitized cells were incubated at 37°C for 45 min. Cells were the washed twice with PBS and centrifuged for 10 min at 1,500 rpm.

LLO_{91-99} sensitized cells were then stained with 5 μM CFSE (in PBS) at $5x10^7$ cells per ml CFSE solution. Control cells were similarly stained but with 0.5 μM CFSE. Cells were incubated at 37°C for 10 min, complete IMDM was added and a last incubation step for 5 min on ice was performed. Cells were then centrifuged for 10 min at 1,500 rpm and washed twice with PBS. Each mouse was to be injected with 150 μl cell suspension i.v. Therefore stained cells were resuspended in an appropriate volume of PBS before being pooled. A fraction of the cell suspension was used for confirmatory FACS analysis prior to i.v. injection.

5 h after injection mice were bled from the tail vein and blood collected in Eppendorf tubes containing 50 μl heparin (Ratiopharm, Ulm, Germany). Blood/heparin mix was diluted with 1 ml PBS and overlayed over 1 ml Ficoll-Paque (Amersham Biosciences, Freiburg, Germany). Samples were centrifuged at room temperature for 20 min at 1,800 rpm without brake. Lymphoid/myeloid cell fraction was collected, washed once in PBS and centrifuged for 10 min at 1,800 rpm. Supernatants were aspirated, cell samples resuspended in 100-200 μl FACS buffer and finally analysed by flow cytometry.

20 h after injecting CFSE labeled cells, mice were sacrificed with CO_2 and heart blood collected. Samples were treated like 5 h samples above.

Control ratios of control cells (CFSE low) and LLO_{91-99} sensitized cells (CFSE high) should be 50:50 (in percent). When a cytotoxic reaction occurs, sensitized cells are killed and therefore the ratio will change. Specific cytotoxic killing can thus be determined.

2.16 Tetramer and intracellular cytokine stainings

WT and IFN-$\beta^{-/-}$ BALB/c female mice aged 8-12 weeks were infected with *L.monocytogenes* as described, in a primary immunization. Control mice were intravenously injected with PBS. At day 35 after the primary immunization, remaining mice were given a second immunization, this time with $1x10^5$ *L.monocytogenes*. At days 3 and 7 after primary immunization, and at days 2 and 5 after secondary immunization, three mice per group were sacrificed and spleens were collected and homogenized through a 100 μm filter. Homogenates were transferred to 15 ml Falcon tubes and centrifuged for 7 min at 1,500 rpm. Supernatants were discarded and pellets resuspended in 5 ml ACT-buffer for lysis of erythrocytes. After 7 min, tubes were filled with complete IMDM and samples were centrifuged as before.

Supernatants were discarded, pellets resuspended in 10 ml complete IMDM and samples filtered into new Falcon tubes. Viable cells were counted using trypan blue exclusion. Intracellular cytokine stainings (ICS) were now started and tetramer stainings were carried out during incubation steps of the ICS procedure.

2.16.1 Intracellular cytokine staining

Splenocytes were plated out in 24-well cell culture plates at $2x10^7$ cells per well in a total volume of 2 ml. Peptide stimuli (LLO_{91-99}, $p60_{217-225}$, H2M3 restricted synthetic f-MIGWIIA) were added to an end concentration of 1 µg/ml, while 50 µl heat-killed *L.monocytogenes* (HKLM) was added. Plates were gently swirled and incubated for 2 h at 37°C.
An ICS-kit (BD, Heidelberg, Germany) was used for the remainder of the procedure and the manufacturer's protocol followed with only minor deviations. Briefly, Golgi plug was added to peptide- and HKLM stimulated samples after the 2 h incubation period. Splenocytes were incubated for an additional 3 h at 37°C in the presence of Golgi plug. Cells were then collected, washed once in FACS buffer and centrifuged for 7 minutes at 1,500 rpm. Samples were resuspended in 100 µl FACS buffer containing 2 µg/ml ethidium monoazide bromide (EMA; Molecular Probes, Leiden, The Netherlands) and Fc-receptor antibody (CD16/32; BD, Heidelberg, Germany). Samples were incubated on ice in the dark for 20 min. 200 ml FACS buffer was added and samples kept on ice and under bright light for 10 min to stop EMA reaction. Samples were washed once in FACS buffer and centrifuged for 3 min at 4°C and at 1,500 rpm.
CD8 and CD62L antibodies were added to samples and stained for 20 min on ice in the dark. Samples were washed 3 times in FACS buffer and centrifuged as before. 100 µl Cytofix-perm solution was added and samples incubated as for antibody staining. 100 µl PermWash diluted in H_2O was added, samples centrifuged and additionally washed twice in PermWash.
Antibodies against IFN-γ and TNF-α from the ICS-kit were added to samples in PermWash and incubated for 30 min on ice and in the dark. Samples were then centrifuged as before and washed twice in PermWash. Finally, samples were resuspended in FACS-buffer and analysed on a FACSCalibur. Gatings were set for $EMA^-CD8^+CD62L^-$ cells and numbers of these cells positive for IFN-γ and TNF-α were calculated.

2.16.2 Tetramer stainings

PE-conjugated LLO_{91-99}, $p60_{217-225}$ and H2M3 restricted synthetic f-MIGWIIA specific tetramers were kindly provided by Prof. Dr. med. Dirk Busch (TU, Munich, Germany).
A total of $6x10^6$ splenocytes/ mouse were transferred to Falcon tubes, and centrifuged for 7 min at 1,500 rpm. Supernatant was discarded and 250 µl FACS buffer containing 2 µg/ml ethidium monoazide bromide (EMA; Molecular Probes, Leiden, The Netherlands) and Fc-receptor antibody (CD16/32; BD, Heidelberg, Germany) was added to each Falcon tube. Cells were incubate for 20 min on ice and in dark. FACS-buffer was added to a total volume of 500 µl and cells were aliquoted in 96-well plates (100 µl/well). Plates were kept on ice and put under bright light for 10 min to stop EMA reaction. Another 100 µl of FACS-buffer was added and plates centrifuged as above. CD8 and CD62L antibodies were added to a mix with the specific tetramers and added to cells. Plates were incubated for 45-60 min on ice in dark. 150 µl FACS-buffer was added and centrifuged as above. Finally, cells were washed twice resuspended in FACS-buffer and analysed on a FACSCalibur. Gatings were set for EMA^- $CD8^+CD62L^-$ cells and numbers of these cells positive for the PE-conjugated LLO_{91-99}, $p60_{217-225}$ and H2M3 tetramers were calculated.

2.17 Induction of acute colitis

Mice were given different concentrations of DSS, dextran sulfate sodium (M.W.=36,000-50,000; MP Biomedicals, Eschwege, Germany) dissolved in autoclaved water *ad libitum* for 7 days. This induced an acute intestinal inflammation.

2.17.1 Clinical assessment of colitis

Starting one day before DSS was administered, mice were weighed daily and monitored for rectal bleeding and diarrhea. Weight loss was described in percent of starting weight.
Rectal bleeding/diarrhea was visually scored by examining the anal area of mice. No rectal bleeding or diarrhea was scored as 0. Dried blood around the anus or a swollen, sore looking rectal area were scored as 1. Diarrhea was scored as 1 when mice had dried fecal products adherent to skin around rectal area, or when stool consisted of pasty and semiformed stools. Maximum score was 2.
Disease activity index was calculated by adding the weight loss in grams and the score for rectal bleeding/diarrhea.

Colon length was mesured after sacrificing mice with CO^2. Measurement started at the part most proximal to the caecum and ended at the most distal part at the rectum.

2.17.2 Flow cytometric analysis of mesenteric lymph nodes

Prior to collecting the colons after sacrificing mice, mesenteric lymph nodes (MLNs) were isolated from one WT and one IFN-$\beta^{-/-}$ mouse. MLNs were pooled and homogenized through a 100 μm nylon mesh. Cell suspensions were washed in PBS and total cell number determined by trypan blue exclusion. Cells were resuspended in FACS-buffer and stained for CD3, CD25, CD4 and CD69, then analysed be flow cytometry.

3 Results

The type I IFNs exert multiple and sometimes complex influences in the body. IFN-$\beta^{-/-}$ mice were therefore used as a way to test type I IFN function in different model systems in order to establish a basis for further molecular studies.

3.1 Analysis of the early kinetics and hierarchy of type I IFNs in virus infected fibroblasts

Previous studies have proposed a hierarchy of temporal expression within the type I IFN gene family (Juang et al., 1998; Marie et al., 1998). IFN-β and IFN-α4 are so called early responders whose transcription is promoted by the transcription factor IRF-3. IFN-non-α4s, the enhancers of which do not contain IRF-3 binding sites, are delayed responders and require activated IRF-7 for transcription. IRF-7, unlike IRF-3, is not constitutively expressed, and its expression is induced by signals from the type I IFN receptor in a positive feedback loop (Sato et al., 1998).

Despite of their shared responsiveness to IRF-3, a further hierarchy was revealed between IFN-β and IFN-α4 in IFN-$\beta^{-/-}$ mice (Deonarain et al., 2000; Erlandsson et al., 1998). In primary embryonic fibroblasts, no IFN-α, including IFN-α4, was induced in primary embryonic fibroblasts (MEF) upon infection with Sendai virus (SeV) (Erlandsson et al., 1998). In contrast, using immortalized fibroblasts from mice that were unable to signal via the type I IFN receptor, it was shown that IFN-α4 can be induced without previous expression of IFN-β (Marie et al., 1998). To directly examine whether cellular immortalization influences the expression pattern of type I IFNs in response to a virus infection, the expression patterns of primary MEFs and muirne adult fibroblasts (MAFs) were compared to those of immortlaized MEFs and MAFs.

3.1.1 Early kinetics and hierarchy of type I IFNs

First the kinetics and temporal hierarchy of type I IFN expression in primary WT, IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ MEFs were studied by RT-PCR. IFN-β was induced in WT MEFs early after virus infection (Fig. 3.1A), and IFN-α4, as well as IFN-non-α4, was detected 6 h after induction. In contrast, MEFs deficient in IFN-β showed a complete absence of IFN-α

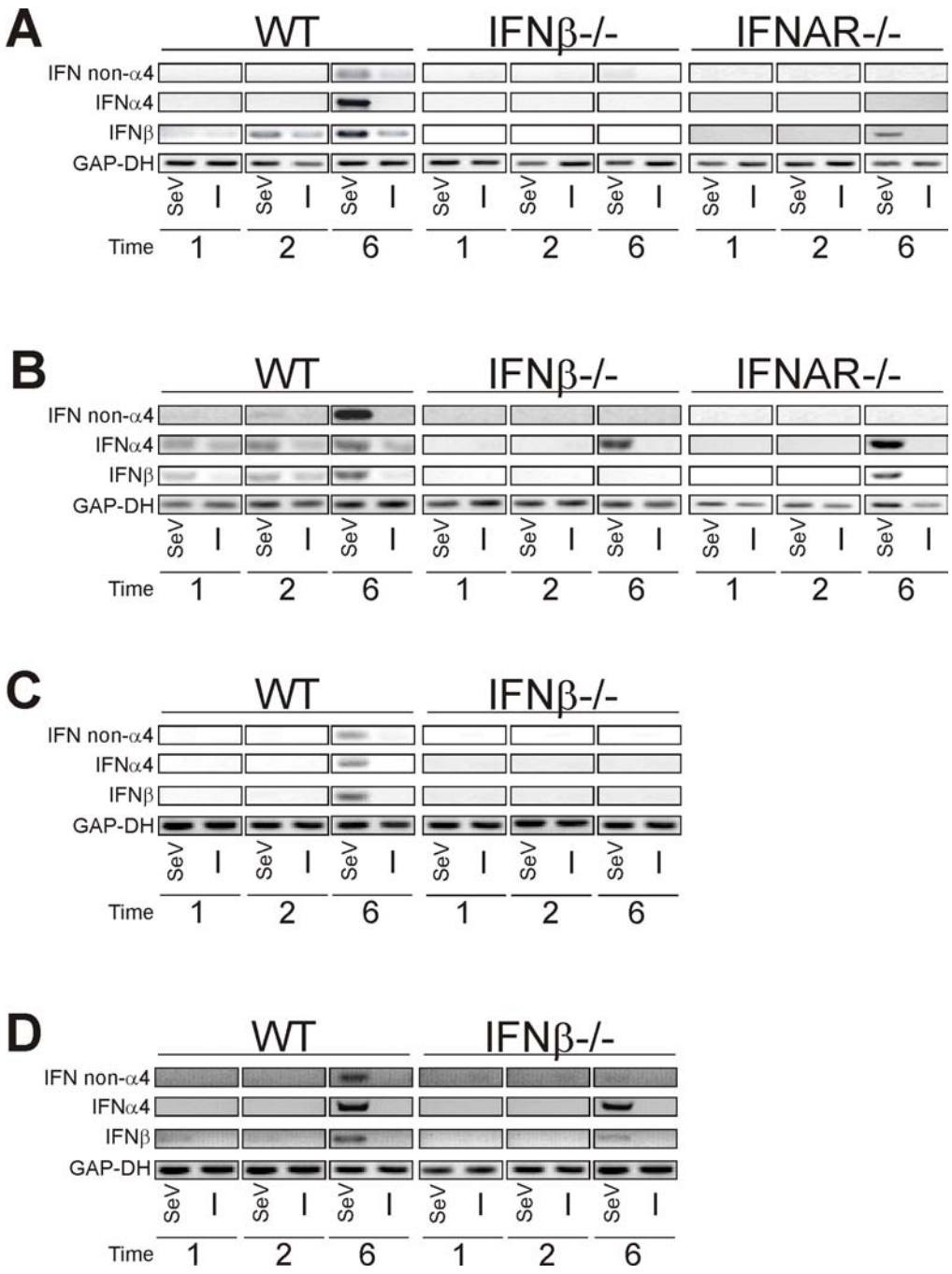


Fig. 3.1*: *Expression of early and late type I IFN mRNA in primary and immortalized MEFs and MAFs after Sendai virus infection. ***A)*** *Primary MEFs,* ***B)*** *Immortalized MEFs,* ***C)*** *Primary MAFs,* ***D)*** *Immortalized MAFs. RT-PCRs were performed for 30 (IFN-β), 34 (IFN-α4) or 36 cycles (IFN-non-α4). GAP-DH was used as a positive control (26 cycles). No signal was detected when omitting the reverse transcriptase (not shown). Time denoted as hours post infection.*

production, as previously observed (Erlandsson et al., 1998). A similar pattern of IFN expression hierarchy was also observed for primary WT and IFN-$\beta^{-/-}$ MAFs (Fig. 3.1C).

These experiments were repeated using the same MEFs and MAFs after immortalisation. In contrast to the results derived from the primary fibroblasts, all immortalized cells were able to produce IFN-α4 independently of signals induced by IFN-β (Fig. 3.1B and D). However, no IFN-non-α4 was detected in immortalized IFN-$\beta^{-/-}$ MEFs and MAFs under these conditions, stressing the importance of IFN-β as the dominant type I IFN in primary as well as in immortalized fibroblasts.
A weak IFN-β band is observed in immortalized IFN-$\beta^{-/-}$ MAFs (Fig. 3.1D). It is unclear why, under some conditions, IFN-β can be detected despite the inactivation of the IFN-β gene by the insertion of a neo cassette between the promoter and the residual IFN-β gene. Nevertheless, no translation is possible from this transcribed mRNA.

3.1.2 Induction of IRF-7 after virus infection

Next, since the transciption factor IRF-7 is requisite in the regulation of many IFN responses to pathogens, the expression pattern of IRF-7 after virus infection was studied. In primary WT MEFs and MAFs, expression of IRF-7 mRNA was detected 6 h after virus infection. No IRF-7 was detected in fibroblasts derived from IFN-$\beta^{-/-}$ or IFNAR$^{-/-}$ mice (Fig. 3.2A and C), consistent with the absence of IFN-α4 expression in these cells.
Interestingly, IRF-7 was constitutively expressed in immortalized WT MEFs and MAFs and increased only slightly after virus infection (Fig. 3.2B and D). In contrast, IRF-7 mRNA was undetectable in both IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ MEFs, while virus infection of IFN-$\beta^{-/-}$ MAFs resulted in detectable expression of IRF-7 after 6 h. This is consistent with the limited IFN-α4 production at that time point (Fig. 3.2D). However, no IFN-non-α4 mRNA was observed in these cells.

3.1.3 Type I IFN secretion after virus induction

To evaluate whether the phenotype of the fibroblast altered the amount of type I IFN secreted, supernatants from infected and uninfected MEFs and MAFs were tested using a bioassay. The amounts of type I IFN secreted by primary MEFs and MAFs 1 and 2 h after infection were close to the level of detection (Fig. 3.3A and C). In contrast, 6 h after infection with SeV,

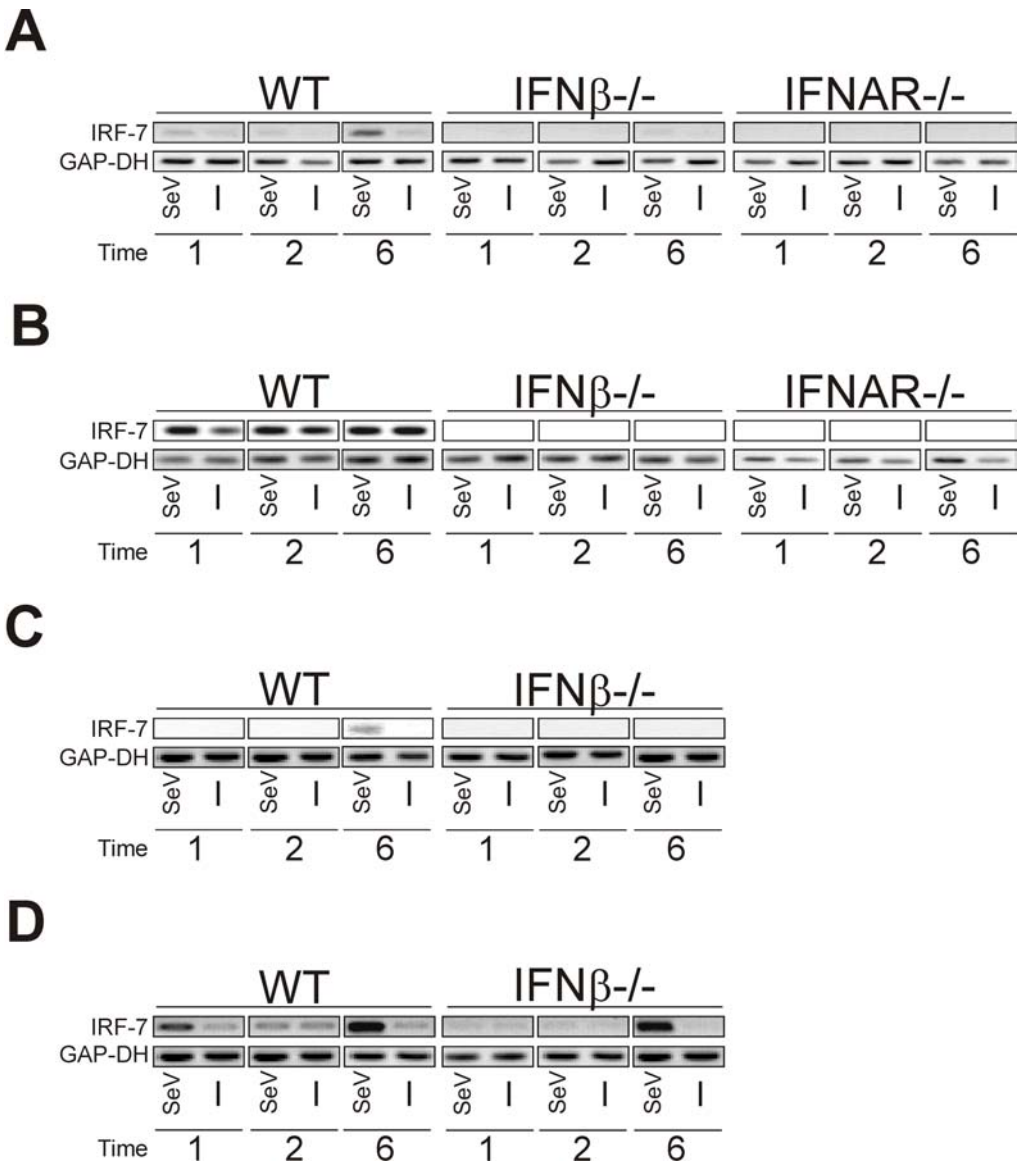


Fig. 3.2: Expression of IRF-7 mRNA in primary and immortalized MEFs and MAFs after Sendai virus infection. ***A)*** *Primary MEFs,* ***B)*** *Immortalized MEFs,* ***C)*** *Primary MAFs,* ***D)*** *Immortalized MAFs. Conditions were as described in Fig. 3.1. Time denoted as hours post infection.*

supernatants from WT MEFs and MAFs contained increased levels of type I IFNs while the levels in IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cells remained low.

Immortalized MEFs and MAFs secreted significantly more type I IFN in these assays when compared with primary MEF and MAF cultures (Fig. 3.3A vs B, C vs D). However, levels secreted from both IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ cells remained constant or even decreased by 6 h post infection while WT cells were able to mount a strong response to infection reflected by increased levels of secreted type I IFN. This result confirms the gene expression data and

highlights the essential role of IFN-β in the regulation of type I IFN responses to viral infections.

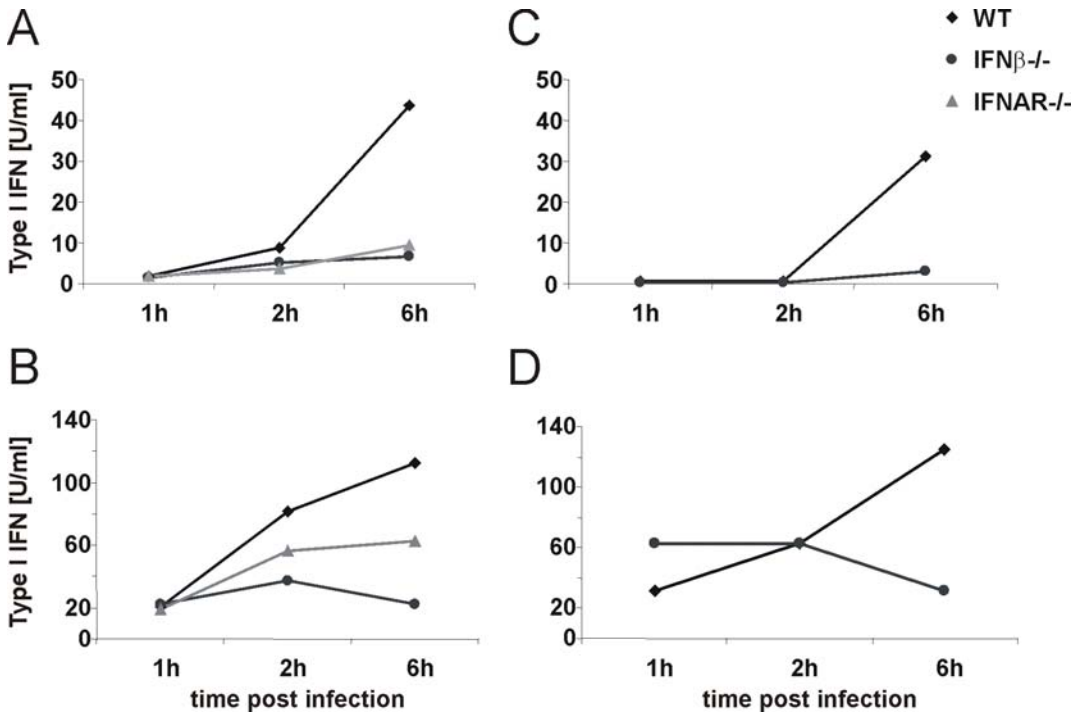


Fig. 3.3: Analysis of secreted type I IFNs in the supernatants of primary and immortalized MEFs and MAFs after SeV infection*. **A)** Primary MEFs, **B)** Immortalized MEFs, **C)** Primary MAFs, **D)** Immortalized MAFs.*

3.1.4 Virus load in primary and immortalized MEFs

Since the levels of secreted type I IFN were different in immortalized and primary fibroblasts it seemed appropriate to evaluate the virus load in the different fibroblasts. To this end viral specific NP (SeVNP) RNA was determined by RT-PCR. SeVNP expression was higher in IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ primary as well as immortalized MEFs, when compared to the WT fibroblasts (Fig. 4). Interestingly, the immortalized MEFs had a higher virus load than their primary counterparts, reflecting the increase in secreted type I IFN in these fibroblasts previously observed. These data confirm the observed lower levels of type I IFN in fibroblasts unable to signal via IFN-β. This again indicates the special role of IFN-β as the expression of IFN-α4 apparently is not of sufficient strength to significantly control the virus infection.

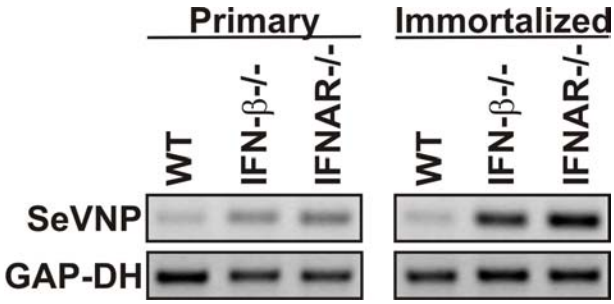


Fig. 3.4: PCR analysis of virus loads in embryonic fibroblasts 6 h after SeV infection*. RNA levels were normalized with GAP-DH and virus loads were determined in primary and immortalized MEFs by RT-PCR analysis of SeVNP expression.*

3.2 Influence of IFN-β on fibroblast proliferation

During the above described experiments, it was noticed that WT fibroblasts had a tendency to grow to confluency slower than IFN-$\beta^{-/-}$ fibroblasts after passaging. IFNs are known to effectively inhibit the growth of various untransformed and transformed cells (Stark et al., 1998), although cells in culture have varying degrees of sensitivity to the antiproliferative activity of type I IFNs. In addition, a considerable amount of ISGs have been shown to have anti-proliferative effects and to be involved, to various degrees, in cell cycle control. Most of these studies, though, involve either exogenously added IFN or overexpression of various ISGs.

3.2.1 IFN-$\beta^{-/-}$ fibroblasts initiate proliferation quicker than WT fibroblasts

To elucidate whether or not there might be an intrinsic activity of type I IFN that might affect proliferatory capacity in fibroblasts, WT and IFN-$\beta^{-/-}$ immortalized MEFs were seeded out at different starting densities. Fibroblasts were then counted at different time points and growth curves were compared. At the lowest starting concentration of immortalized MEFs (100 cells/well), IFN-$\beta^{-/-}$ fibroblasts showed a dramtic increase in proliferation after 7 days while in WT fibroblasts hardly any proliferation whatsoever could be observed, even at the end of the experiment at day 10 (Fig. 3.5A).

Starting at 1,000 cells/well, the IFN-$\beta^{-/-}$ fibroblasts showed an earlier starting point of proliferation as compared to the WT fibroblasts, though their growth rate had a similar inclination once proliferation was initiated (Fig. 3.5B). Growth curves of WT and IFN-$\beta^{-/-}$ fibroblasts seeded out at a high density (10,000 cells/well) did not detectably differ (Fig. 3.5C).

Therefore, depriving fibroblasts of cell to cell contact after seeding seems to activate the type I IFN system and affects the initial point of when fibroblasts regain their proliferatory capacity. Lack of IFN-β apparently does not affect the growth rate of fibroblasts.

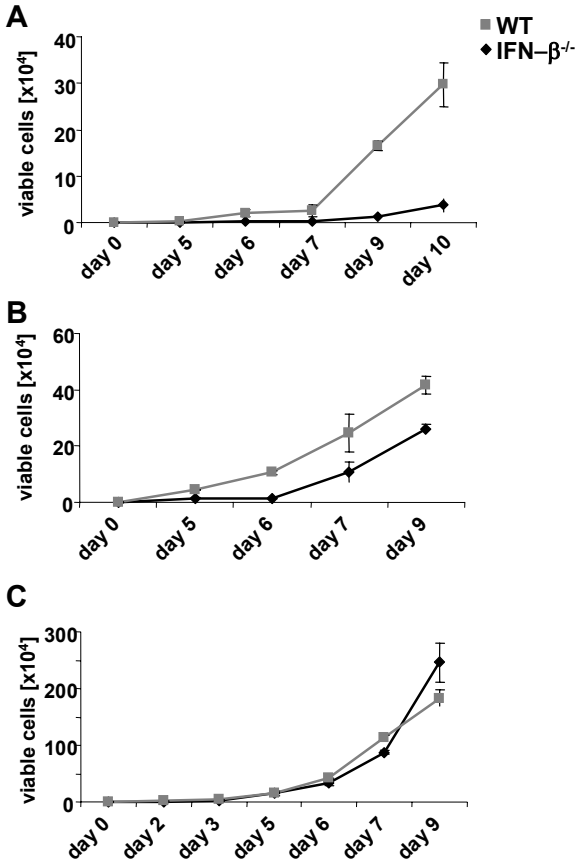


Fig. 3.5: Initiation of fibroblast proliferation is cell density dependent*. Immortalized MEFs were seeded in 24-well cell culture plates. Different starting amounts of cells were seeded: 100 cells/well (**A**), 1,000 cells/well (**B**) or 10,000 cells/well (**C**).*

3.2.2 The observed earlier initiation of proliferation in IFN-β$^{-/-}$ fibroblasts is not a clonal phenomenon

To exclude that the above observation was a clonal phenomenon of the particular preparation of MEFs, a fresh batch of immortalized MEFs was prepared and MEFs from IFNAR$^{-/-}$ mice were now also included. Immortalized MEFs were seeded in 6-well culture plates. IFN-β$^{-/-}$ and IFNAR$^{-/-}$ fibroblasts showed an earlier onset of growth as compared to WT fibroblasts (Fig. 3.6A). Continued culturing of the fibroblasts showed an undiminished proliferatory

capacity in the IFN- $\beta^{-/-}$ MEFs, while in the IFNAR$^{-/-}$ MEF culture the increase in cell growth stalled at the end of the experiment, just when WT MEFs started proliferating (Fig. 3.6B).

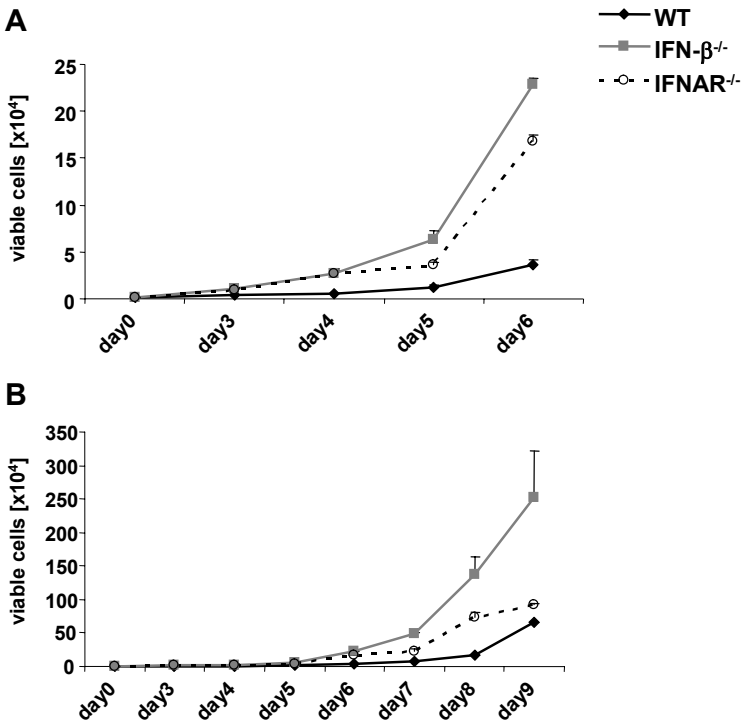


Fig. 3.6: Initiation of growth of immortalized WT, IFN-β$^{-/-}$ and IFNAR$^{-/-}$ MEFs. *Cultures in (**A**) and (**B**) are identical. (**A**) Displays results for the first 6 days of culture. Y-axis is of a different scale to better demonstrate initiation of proliferation. A density of 1,000 cells/well was used in these experiments and cells were seeded out in 6-well cell culture plates.*

3.2.3 Paracrine delay of growth initiation

Two explanations were possible for the observed differences in initiation of proliferation between WT and IFN-$\beta^{-/-}$ fibroblasts. One is that IFN-β could be produced by WT fibroblasts at confluency in the preceding culture, leading to growth arrest when fibroblasts are seeded at low densities. Alternatively, cells in low density cultures could themselves produce IFN-β that retards initiation of proliferation. The latter explanation was tested by seeding a mixture of WT and IFN-$\beta^{-/-}$ fibroblasts at low density. Spontaneous production of IFN-β by WT fibroblasts in the mixture should revert the growth curve to a WT curve. Again, immortalized MEFs were seeded in 6-well plates, either WT cells or IFN-$\beta^{-/-}$ fibroblasts alone, or a 50:50 mixture of WT and IFN-$\beta^{-/-}$ cells. As seen before, the IFN-$\beta^{-/-}$ fibroblasts initiate growth

several days earlier than WT fibroblasts. However, the mixture of WT and IFN-$\beta^{-/-}$ fibroblast reverted the growth curve, which now almost exactly followed the curve of the WT immortalized MEFs (Fig. 3.8). This indicates that WT cells secrete IFN-β that acts on neighbouring IFN-$\beta^{-/-}$ cells in a paracrine manner, retarding the growth of these IFN-β deficient fibroblasts.

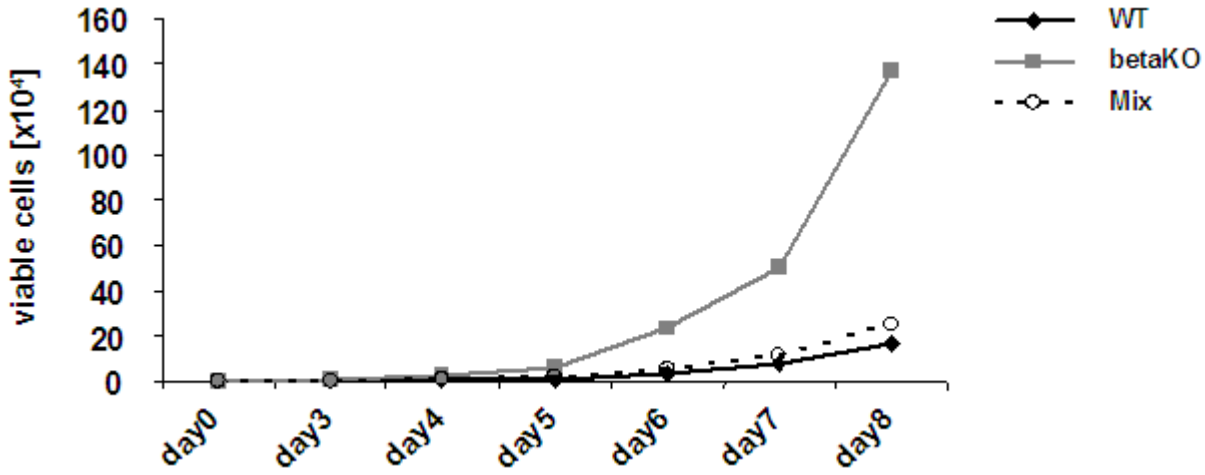


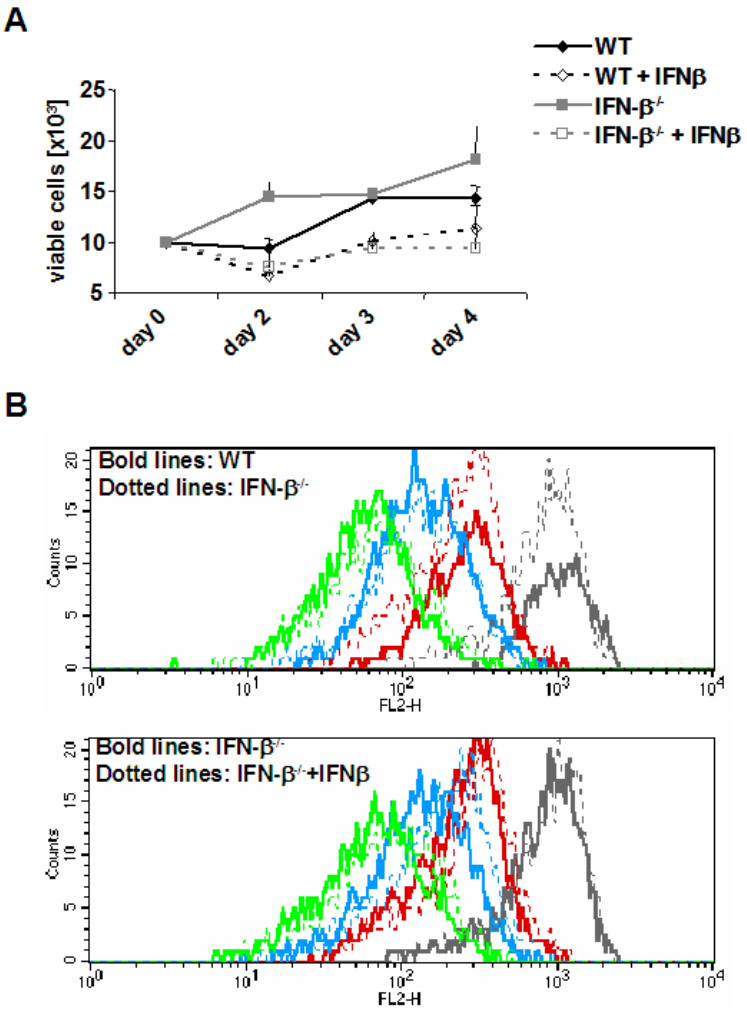
Fig. 3.7: Paracrine inhibition of fibroblast growth by IFN-β*. WT or IFN-$\beta^{-/-}$ MEFs alone, or a 50:50 mixture of WT and IFN-$\beta^{-/-}$ MEFs were seeded at 1,000 cells/well in 6-well cell culture plates. MEFs were counted at different time points and a growth curve was calculated. Mixing WT and IFN-$\beta^{-/-}$ cells reverts the growth curve to almost exactly follow the curve of the WT immortalized MEFs, while the IFN-$\beta^{-/-}$ cells alone freely proliferate.*

3.2.4 Initiation of proliferation and growth of adult fibroblasts at high cell densities is not influenced by the lack of IFN-β

Cell growth experiments were also carried out with WT and IFN-$\beta^{-/-}$ primary adult fibroblasts. To this end, 10,000 MAFS/well were seeded in 6-well plates.

Primary IFN-$\beta^{-/-}$ MAFs exhibited an earlier onset of proliferation than WT MAFs(Fig. 3.8A). This difference did not persist and already at day 3 WT MAF numbers were equal to those of the IFN-$\beta^{-/-}$ MAFs (Fig. 3.8A). In addition, recombinant murine IFN-β (rmIFN-β) was added to both WT and IFN-$\beta^{-/-}$ MAFs to find out whether or not IFN-β exerts a direct antiproliferative effect on these cells. However, adding rmIFN-β decreased the growth of MAFs only slightly.

To find whether this effect is due to delayed proliferation of alls cells or only subpopulations, he above findings were validated by the use of a CFSE-assay. In this assay, proliferation of



Mean fluoresence

	Day1	Day2	Day3	Day4
WT	1031+/-3	292+/-3	162+/-2	73+/-1
WT+IFN-β	1084+/-9	366+/-12	196+/-0	86+/-2
IFN-β-/-	852+/-5	256+/-6	181+/-25	81+/-1
IFN-β-/- +IFN-β	919+/-0	306+/-5	205+/-0	100+/-0

Fig. 3.8: Analysis of WT and IFN-β$^{-/-}$ fibroblast proliferation in the presence of rmIFN-β. *Growth curves were repeated with primary MAFs (**A**). 10,000 MAFs were seeded in 6-well plates and counted at different time points. **B**) CFSE-labeled MAFs. Mean fluorescent intensity was measured at different time points. Upper graph depicts the relationship between WT and IFN-β$^{-/-}$ MAFs, and the lower graph the relationship between untreated IFN-β$^{-/-}$ MAFs and IFN-β$^{-/-}$ MAFs treated with 100 U /ml of rmIFN-β.. The measured mean fluorescence from two independent experiments is summarized in the table on the right, and histograms are taken from one representative experiment.*

cells stained with the fluorescent dye CFSE can be followed, as the dye is diluted upon cell division and and the intensity of fluorescence accordingly decreases. Interestingly, fluorescence decreased at similar rates both WT and IFN-β$^{-/-}$ MAFs (Fig. 3.8B upper panel) and the same number of cell divisions seemed to occur. The same was true for IFN-β$^{-/-}$ MAFs

that were treated with rmIFN-β. Also, treated cells exhibited a decrease in fluorescence similar to that of the IFN-β deficient MAFs (Fig. 3.7B lower panel). Interestingly, despite the cell growth detected in the CFSE assay, hardly any increase in cell numbers were observed (Fig. 3.8A). This might be explained by an increase in apoptosis caused by rmIFN-β and should be further studied.

In addition, MAFs at high densities were also analysed for their cell cycle progression. Previous studies had shown that type I IFNs can induce G1 phase arrest and that activated IRF-5 can induce G2/M phase arrest. Therefore, MAFs were cultured in 75 cm² cell culture flasks at 100,000 cells per flask in either unsupplemented medium or medium supplemented with 100 U/ml rmIFN-β. WT and IFN-$\beta^{-/-}$ fibroblasts were also analyzed at day 0, i.e. when fibroblasts were harvested from a confluent culture before being recultured under the described conditions. The cells used were from the same culture that were also analysed in Fig. 3.7, and the cell cycle analysis results are shown in Fig. 3.9.

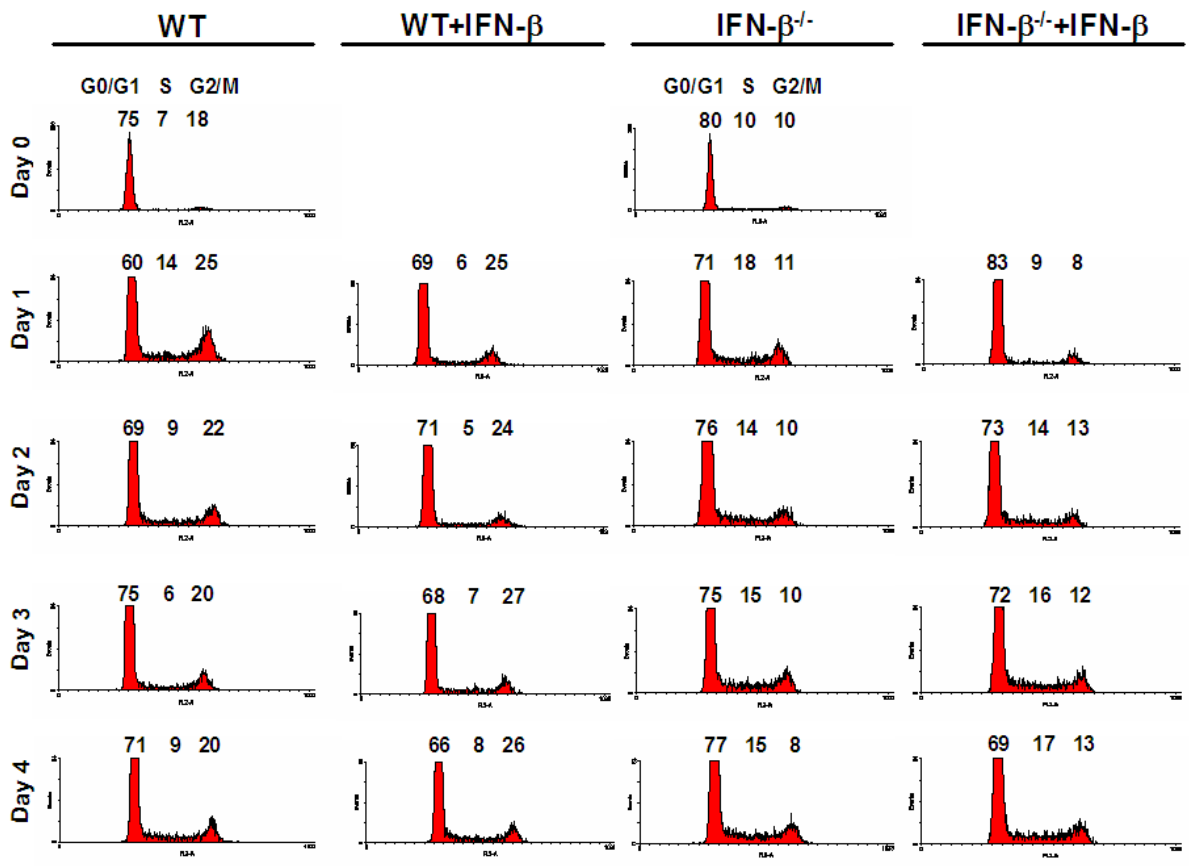


Fig. 3.9: Cell cycle analysis of murine adult fibroblasts*. Graphs show percent of fibroblasts in the different stages of the cell cycle, as determined by PI staining. Experiment was done once.*

Cell cycle analysis showed that WT fibroblasts hardly had any differences in cell cycle profile at day 0 when compared to IFN-$\beta^{-/-}$ fibroblasts. 18% of WT MAFs were in G2/M-phase as compared to 10% of IFN-$\beta^{-/-}$ MAFs. Slight changes are observed during the proliferation time course. The fraction of WT MAFs in G2/M increased slightly to 25% at day 1 and 22% at day 2, while the G2/M fraction in IFN-$\beta^{-/-}$ MAFs remained around 10%. In essence, at onset both WT and IFN-$\beta^{-/-}$ MAFs are in similar cell cycle stages. This might indicate that there is no infleunce on the fibroblasts from the preceeding confluent cultures.
Furthermore, MAFs treated with rmIFN-β have cell cycle profiles that only mildly deviate from untreated MAFs. This fits with data from CFSE labeled MAFs in Fig. 3.7B, where IFN-β treated fibroblasts show a decrease in fluorescence similar to that of untreated MAFs. However, since under these circumstances only a minor increase of cell numbers is observed, IFN-β might increase apoptosis in these cuultures.

3.3 Effect of IFN-β deficiency on bone marrow derived APCs

Previous studies conducted in IFNAR$^{-/-}$, TLR3$^{-/-}$ and MyD88$^{-/-}$ mice (Hoebe et al., 2003) have shown that LPS signals via TLR4 using the Trif- IFNβ- type I IFN-receptor pathway to induce upregulation of costimulatory molecules (UCM). The same pathway, stimulated via TLR3, was also shown to play a role in dsRNA-induced UCM. Therefore it was of interest to study the role of endogenous IFN-β in antigen presenting cells (APCs), in the context of UCM and the capacity of APCs to activate T cell proliferation, after stimulation with virus and different PAMPs.
To this end, macrophages (BMMϕ) and DCs (BMDC) were differentiated from bone marrow of WT and IFN-$\beta^{-/-}$ mice and stimulated with various PAMPs for 24 hours.

3.3.1 Impaired type I IFN secretion in bone marrow derived APCs

BMMϕ and BMDC were stimulated with LTA, LPS, poly I:C or Sendai virus for 24 hours. After this time, type I IFN secretion was measured in an IFN bioassay. Significantly lower amounts of type I IFN in both IFN-$\beta^{-/-}$ BMMϕ and BMDC was detected (Fig. 3.10).
This difference was most dramatic in BMMϕs. In these cells no type I IFN was detected in IFN-β deficient cells after LPS stimulation and only low amounts after stimulation with poly I:C and SeV. In the BMDCs, on the other hand, the differences were not as large, but even so WT cells secreted at least two fold more type I IFN after SeV infection and 4-fold more after poly I:C stimulation. Interestingly, LPS did not stimulate any type I IFN secretion in BMDCs

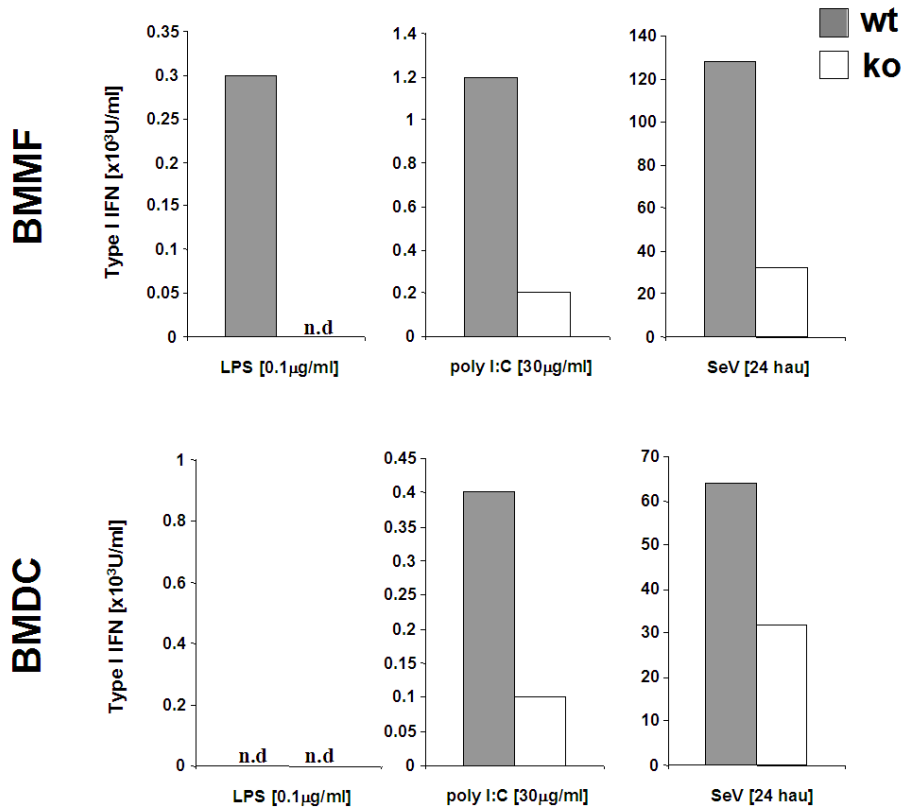


***Fig. 3.10**: **Type I IFN secretion by BMMϕ and BMDC after stimulation with different pathogen associated molecules or Sendai virus for 24 hours**. LTA stimulation did not elicit type I IFN secretion from either cell type. BMMϕ are represented in upper panels and BMDC in lower panels. Note the y-axis scale differences in the different panels. n.d = not detectable.*

3.3.2 IFN-β deficiency affects upregulation of costimulatory molecules on BMMϕs

After poly I:C stimulation, CD86 and ICAM-1 were found to be more upregulated on WT than on IFN-$\beta^{-/-}$ BMMϕs. Other costimulatory molecules, CD40 and CD80, were to a lesser degree affected, though also in this case upregulation was slightly impaired in IFN-$\beta^{-/-}$ BMMϕs. Furthermore, the upregulation of MHC-molecules was also affected in BMMϕs deficient for IFN-β. For the other stimuli used (LTA, LPS and SeV), no consistent differences in the upregulation of the above mentioned molecules could be observed.

Taken together, these data indicate that, at least for some PAMPs, IFN-β is needed for an efficient upregulation of antigen presenting and costimulatory molecules on BMMϕs.

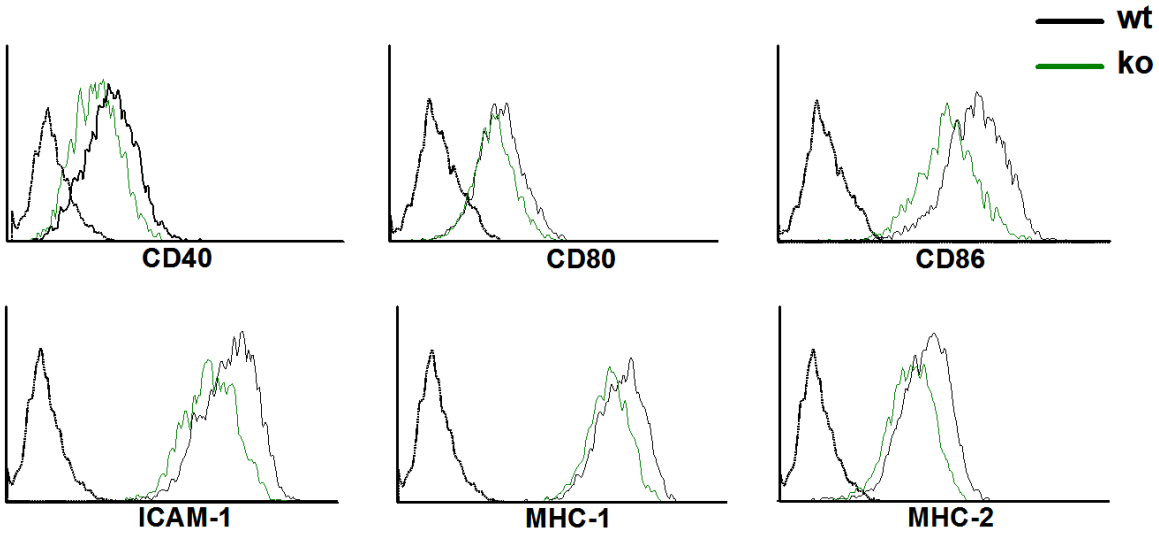


Fig. 3.11: Flow cytometric analysis of BMMϕ stimulated with poly I:C for 24 hours*. IFN-β deficient BMMϕ are less efficient in upregulating CD86 and ICAM-1. CD40 and CD80, two other costimulatory molecules, are only slightly differentially upregulated. Furthermore, IFN-β$^{-/-}$ cells are less efficient in upregulating MHC molecules after poly I:C stimulation. MHC-1 and MHC-2 indicate MHC class I and II, respectively. Black lines are WT, green lines are IFN-β$^{-/-}$ cells and bold black lines are isotype control. Histograms are taken from one representative experiment.*

3.3.3 CD8^{+} T cell stimulatory capacity is impaired in IFN-β deficient BMMϕs, but not affected in BMDCs

Since differences in the upregulation of costimulatory molecules was affected in IFN-β$^{-/-}$ BMMϕ, it was of interest to investigate whether or not this would have any effect on BMMϕ function. Therefore, control BMMϕs or BMMϕs stimulated with various PAMPs for 24 hours were pulsed with HA-peptide. The BMMϕs were then added to peptide specific CL4 T cells to investigate the BMMϕs T cell stimulatory capacity.

Control and LTA stimulated BMMϕs show low T cell stimulatory capacity and there is no difference in the stimulatory capacity between WT and IFN-β deficient BMMϕs (Fig. 3.12). This is consistent with the lack of type I IFN secretion inBMMϕs stimulated with LTA.

Poly I:C and LPS stimulated BMMϕs are more efficient in stimulating T cell proliferation and the stimulatory capacity of IFN-β deficient BMMϕ is much less than that of WT BMMϕ. These differences fit with the data demonstrating impaired upregulation of type I IFNs and of costimulatory molecules on poly I:C stimulated BMMϕs.

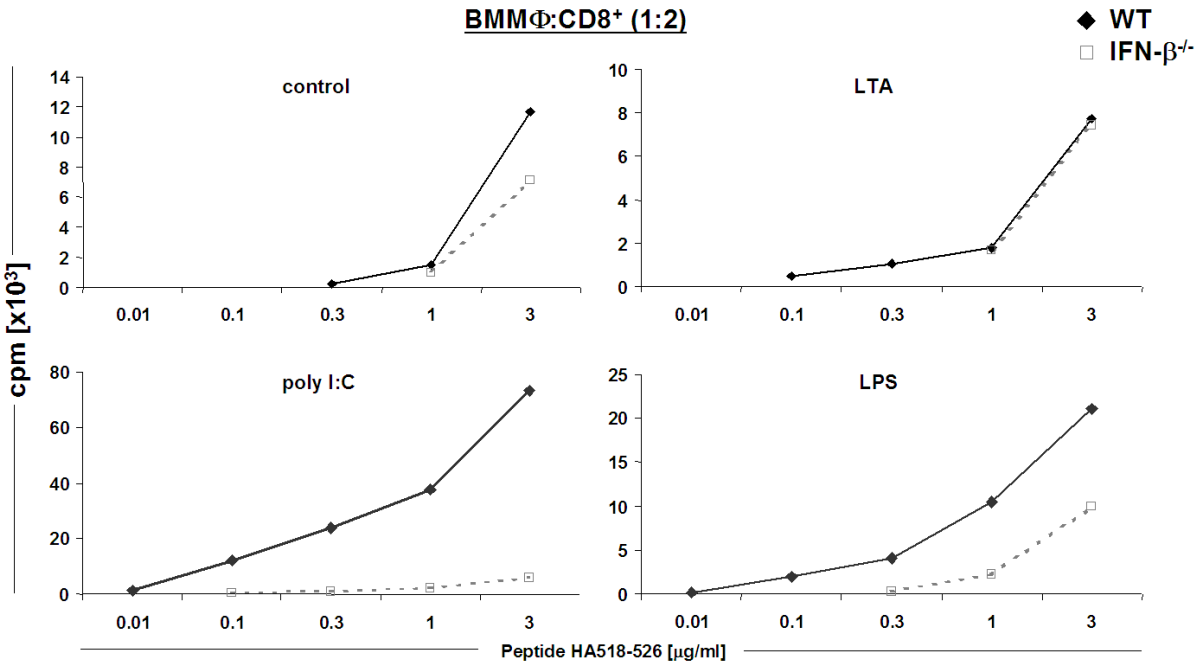


***Fig. 3.12**: **T cell stimulatory capacity of BMMϕ from WT and IFN-β$^{-/-}$ mice after stimulation with varoius PAMPs**. Stimulation with indicated molecules was measured by incubation with CD8^{+} T cells from spleens of CL4 transgenic mice specific for a Influensa virus HA-peptide presented by MHCclass I. Peptide was added at indicated concentrations and [^{3}H]-thymidine uptake was measured as an indication for T cell proliferation. Graphs are taken from one representative experiment, and experiments were repeated twice.*

Furthermore, even though BMDCs had not been investigated for differences in upregulation of costimulatory molecules, it was of interest to study whether the observed differences in the BMMϕs capacity of stimulating T cell proliferation also was true for BMDCs. Therefore, the experiment was repeated with BMDCs.

Overall, BMDCs are more efficient in stimulating T cell proliferation in comparison to BMMF. Though, for BMDCs hardly any differences could be detected in T cell stimulatory capacity between WT and IFN-β deficent cells (Fig. 3.13). The T cell stimulatory capacity was somewhat lower in poly I:C and LPS stimulated IFN-β$^{-/-}$ BMDCs though not significantly so. Either type I IFNs do not play a role in these cells or IFN-β deficiency can be compensated by IFN-α.

These data indicate that IFN-β is not essential for an effective immune response *in vitro,* and that IFN-β deficiency can, to a large extent, be compensated by IFN-α. Nevertheless, the differences in the response of the two antigen presenting cell types to different stimuli emphasises the complexity of the type I IFN system.

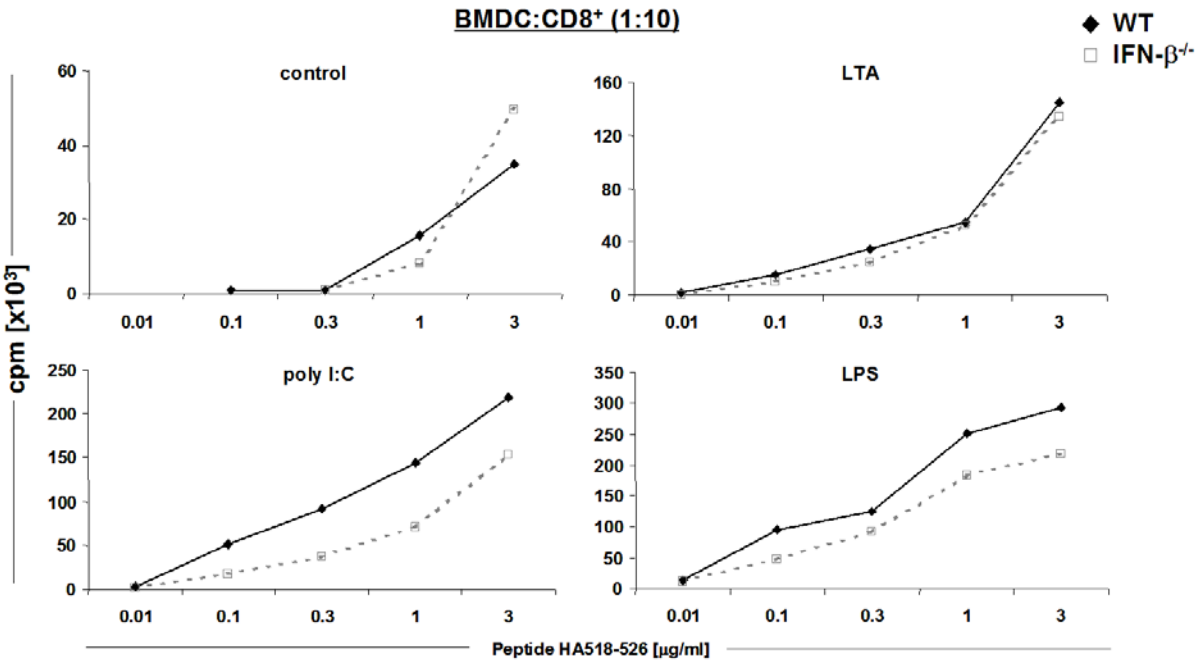


***Fig. 3.13**: **T cell stimulatory capacity of BMDC from WT and IFN-β$^{-/-}$ mice after stimulation with varoius PAMPs**. Stimulation with indicated molecules was measured by incubation with CD8^{+} T cells from spleens of CL4 transgenic mice. Peptide was added at indicated concentrations and [^{3}H]-thymidine uptake was measured as an indication for T cell proliferation. Graphs are taken from one representative experiment.*

3.4 Role of IFN-β in L.monocytogenes infection

In addition to viruses, a large number of microbes and microbial products have been described to trigger production of type I IFNs both *in vivo* and in various cell types *in vitro* (Bogdan, 2000). Furthermore, injecting recombinant type I IFN can protect mice from various microbial challanges. This, together with the reported immunomodulatory effects of type I IFNs (Theofilopoulos et al., 2004), initiated the examination of the susceptibility of IFN-β$^{-/-}$ mice to *L.monocytogenes* infection. Furthermore, adaptive immune responses in the IFN-β$^{-/-}$ mouse in response to Listeria infection were analyzed. Finally, the expression pattern of type I IFNs in different splenic cell populations in response to Listeria infection was examined.

3.4.1 Only minor differences in bacterial loads found in WT and IFN-β deficient mice

To investigate whether endogenously produced IFN-β during a listerial infection plays a role in clearing the infection, WT and IFN-β$^{-/-}$ mice were injected with a sublethal dose of *L.monocytogenes* (2x10³). The infection was followed over a course of four days, and on each

of these days liver and spleen was taken from sacrificed mice and homogenized before being plated out. Bacterial colonies were then counted and compared.

No large differences were noticed in number of colony forming units (CFU) in livers and spleens between WT and IFN-$\beta^{-/-}$ mice (Fig. 3.14). Especially CFUs in spleen were almost identical between WT and IFN-$\beta^{-/-}$ mice, while minor differences were noticed in the liver. On day 3 post infection, the biggest difference could be seen in liver. IFN-$\beta^{-/-}$ mice then have a significantly lower bacterial load than the WT mice, a difference that persists throughout the later time points.

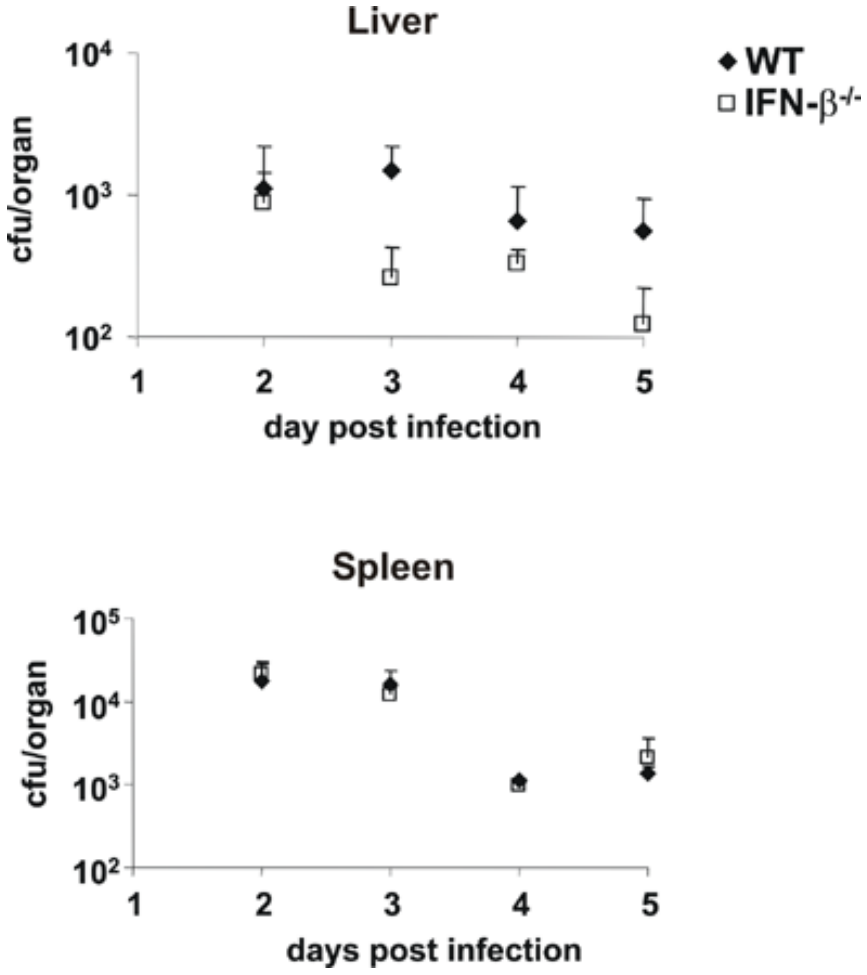


***Fig. 3.14*: Bacterial loads in liver and spleen determined by colony forming units (CFU) from homogenized organs of WT and IFN-$\beta^{-/-}$ mice**. *Mice were infected with 2x10³ L.monocytogenes and organs were collected from sacrificed mice at different days post infection. Organs were then homogenized and plated on bacterial growth plates and incubated over night. The next day CFUs were counted and compared. Graphs are taken from one representative experiment with 3-4 mice per group.*

3.4.2 The *in vivo* cytotoxic response against *L.monocytogenes* is slightly more efficient in IFN-β deficient mice 5 days post infection.

Since type I IFNs are suggested to play a part in immunomodulation and in bridging the innate and adaptive immune systems, it was of interest to investigate the ability of IFN-β deficient mice to mount a cytotoxic response against *L.monocytogenes*. This was especially interesting as a dramatic effect was observed in mice that were deficient for the type I IFN receptor (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). To this end, WT and IFN-$\beta^{-/-}$ mice were infected with a $2x10^3$ *L.monocytogenes*, and the cytotoxic responses measured by an *in vivo* killer assay. Briefly, an aliquot of spleen cells from either naive WT or IFN-$\beta^{-/-}$ mice were sensitized with a *L.monocytogenes* specific peptide (LLO_{91-99}) on different days p.i. Both WT and IFN-β deficient cells were used in the experiment to exclude any influence WT cells with an IFN-β producing capacity might have on the cytotoxic activity of IFN-$\beta^{-/-}$ mice. Another fraction of spleen cells were left untreated. These two fractions were then stained with two different concentrations of CFSE, a fluorescent dye, to facilitate flow cytometric analysis. The cells were then combined and injected into naive control or *L.monocytogenes* immunized mice. *In vivo* cytotoxicity was then measured by isolating blood cells 5 h and 20 h post injection of cells, and analyzing these cells by flow cytometry.

Fig. 3.15A represents a typical histogram chart. In control mice, no cytotoxic reaction occurs and both control cells (low CFSE) and LLO_{91-99} sensitized cells (high CFSE) remain at their initial 50:50 relationship. In *L.monocytogenes* immunized mice the relationship starts changing at day 4 as the LLO_{91-99} sensitized cells are being recognized by immune cells in these mice. Hence the cytotoxic reaction starts and LLO_{91-99} sensitized cells are killed (Fig. 3.15A).

The formula used in order to calculate the actual percentage of killing of LLO_{91-99} sensitized cells is shown in Fig. 3.15B, and the graphs summarizing the experiment are displayed in Figs 3.15C-E.

The *in vivo* cytotoxic killing was analyzed on days 4, 5 and 6 p.i. On day 4 p.i. no difference in cytotoxic activity could be found (Fig. 3.15C) in both WT and IFN-$\beta^{-/-}$ mice. LLO_{91-99} sensitized cells were efficiently killed. No difference was seen at 5 h or 20 h post injection, although killing was much higher at the 20 h time point.

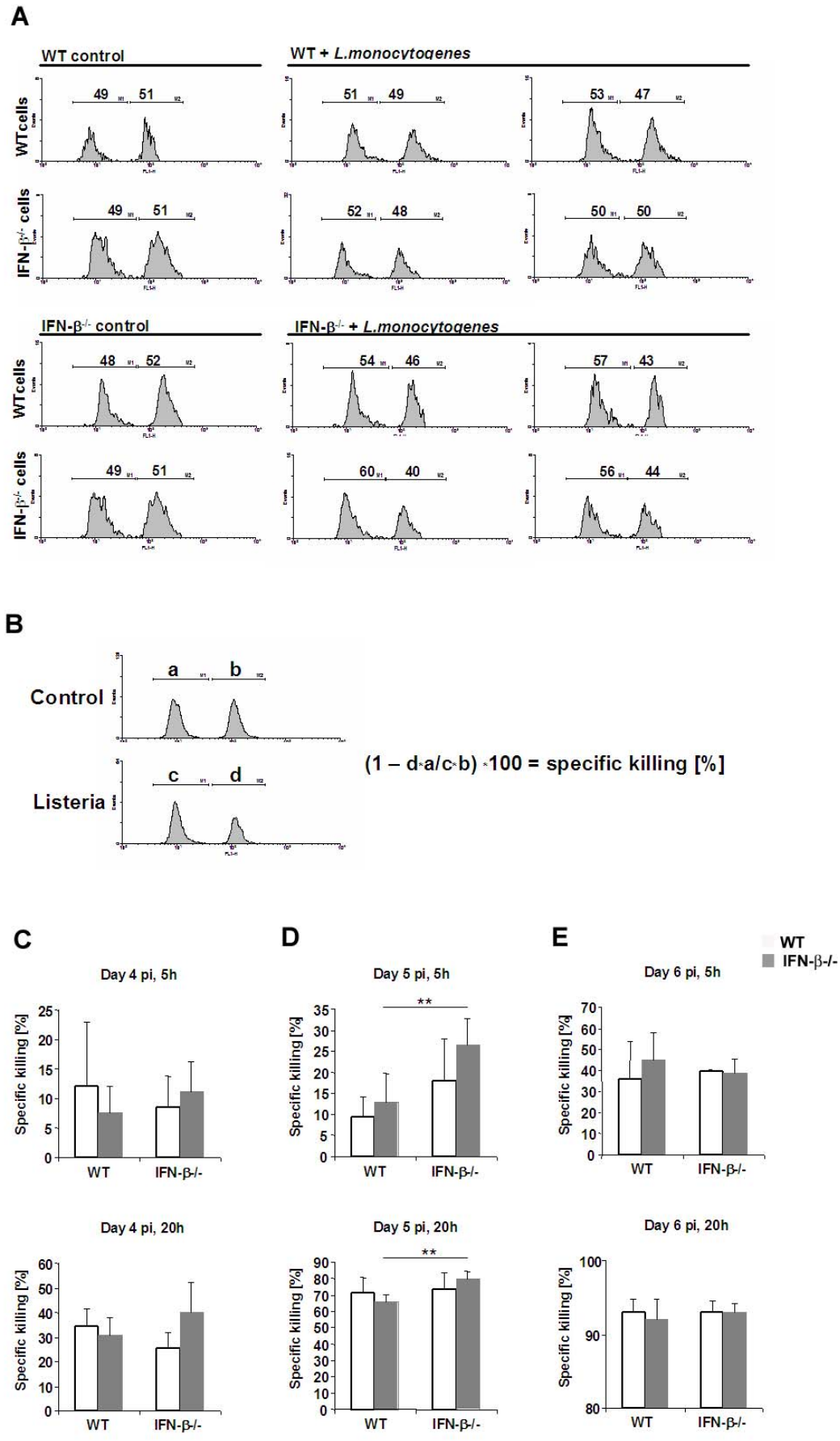

A
WT control
WT + L.monocytogenes
WTcells
IFN-β-/- cells
49 51
51 49
53 47
49 51
52 48
50 50
IFN-β-/- control
IFN-β-/- + L.monocytogenes
48 52
54 46
57 43
49 51
60 40
56 44
B
Control
Listeria
a b
c d
(1 – d×a/c×b) ×100 = specific killing [%]
C
D
E
WT
IFN-β-/-
Day 4 pi, 5h
Day 5 pi, 5h
Day 6 pi, 5h
Day 4 pi, 20h
Day 5 pi, 20h
Day 6 pi, 20h
Specific killing [%]
**
WT
IFN-β-/-

***Fig. 3.15: In vivo cytotoxicity assay in WT and IFN-β deficient mice after L.monocytogenes infection**. **A)** Histogram chart from day 5 p.i. and 5 h after injecting CFSE-labeled cells. Mice immunized with L.monocytogenes show in vivo killing as peaks representing high CFSE concentration are diminishing. **B)** Formula used for calculating psecific killing. **C)** Graphs summarizing histogram charts as specific killing at days 4, 5 and 6 p.i and 5 or 20 h after injecting CFSE-labeled cells. Histogram chart shows one of two experiments at this time point and graphs summarize two independent experiments with two mice in each group. (**p<0.01)*

Interestingly, on day 5 p.i. differences in killing between WT and IFN-$\beta^{-/-}$ mice were observed (Fig. 3.15D). Already at 5 h post injection, LLO_{91-99} sensitized cells were being killed in significantly higher amounts in IFN-$\beta^{-/-}$ mice than in WT mice. This difference was even more obvious in IFN-$\beta^{-/-}$ mice that had been injected with LLO_{91-99} sensitized IFN-β deficient cells. This difference could still be observed 20 h post injection of sensitized cells, though it was not as clear cut at this time point as at 5 h.

Furthermore, the difference in killing activity was no longer observable on day 6 p.i. (Fig. 3.15E), and almost all sensitized cells were killed 20 h post injection.

In summary, it is likely that IFN-β alone does not play a crucial part in priming a cytotoxic response against *L.monocytogenes in vivo*, though at some point during the development of the adaptive immune response IFN-β deficiency facilitates a more efficient cytotoxic *in vivo* response.

3.4.3 No major differences of adaptive immune responses to *L.monocytogenes* infection in IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ mice

To further study eventual impact on the triggering of adaptive immune responses in IFN-β deficient mice, tetramer- and intracellular cytokine stainings (ICS) were performed after *L.monocytogenes* infection. Briefly, IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ mice were infected with $1x10^3$ *L.monocytogenes* and analyzed on day 3 and 7 after infection. Day 3 represents a time point when the adaptive immune responses are in their initial phase and day 7 when they should be at their peak.

Spleen cells were stained with CD8, CD62L and tetramers specific for LLO_{91-99}, $p60_{217-225}$ or H2M3 restricted synthetic f-MIGWIIA peptide(henceforth described as H2M3-peptide) and analysed by flow cytometry. The amount of mature CD8 T cells ($CD8^+$ and $CD62L^{lo}$) were then calculated for IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ mice. On day 3 p.i. no significant differences were seen in mature CD8 cells specific for either LLO_{91-99} or $p60_{217-225}$, though there were more H2M3-peptide specific cells in the IFN-$\beta^{+/-}$ mice (Fig. 3.16A). On day 7 p.i. there were over

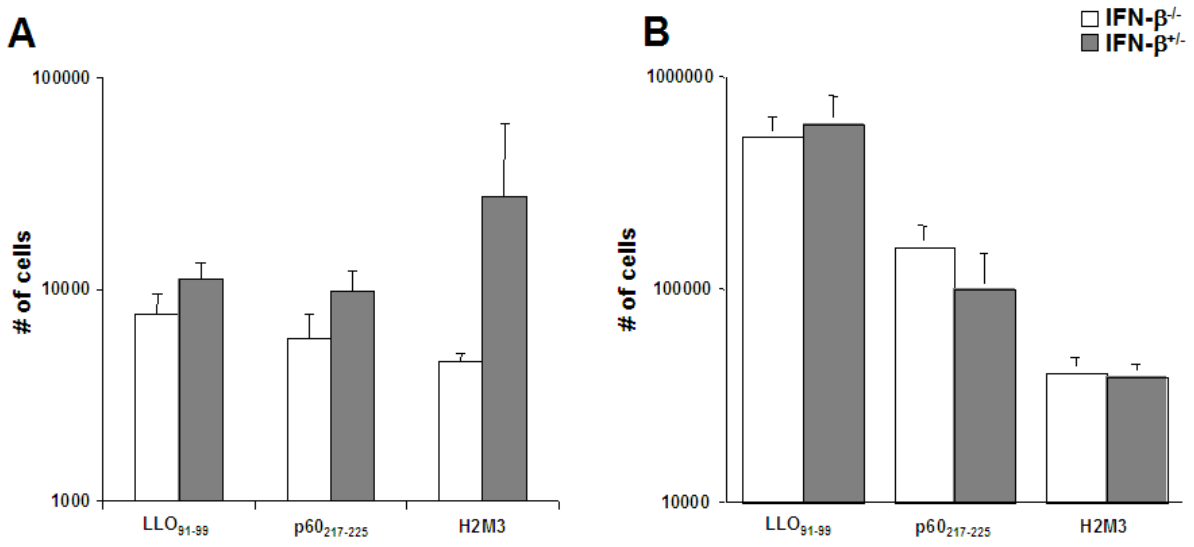


***Fig. 3.16**: **Mature CD8+CD62L^{lo} specific for LLO$_{91-99}$, p60$_{217-225}$ and H2M3-peptide tetramers**. **A)** Day 3 after primary immunization with L.monocytogenes. **B)** Day 7 after primary immunization.*

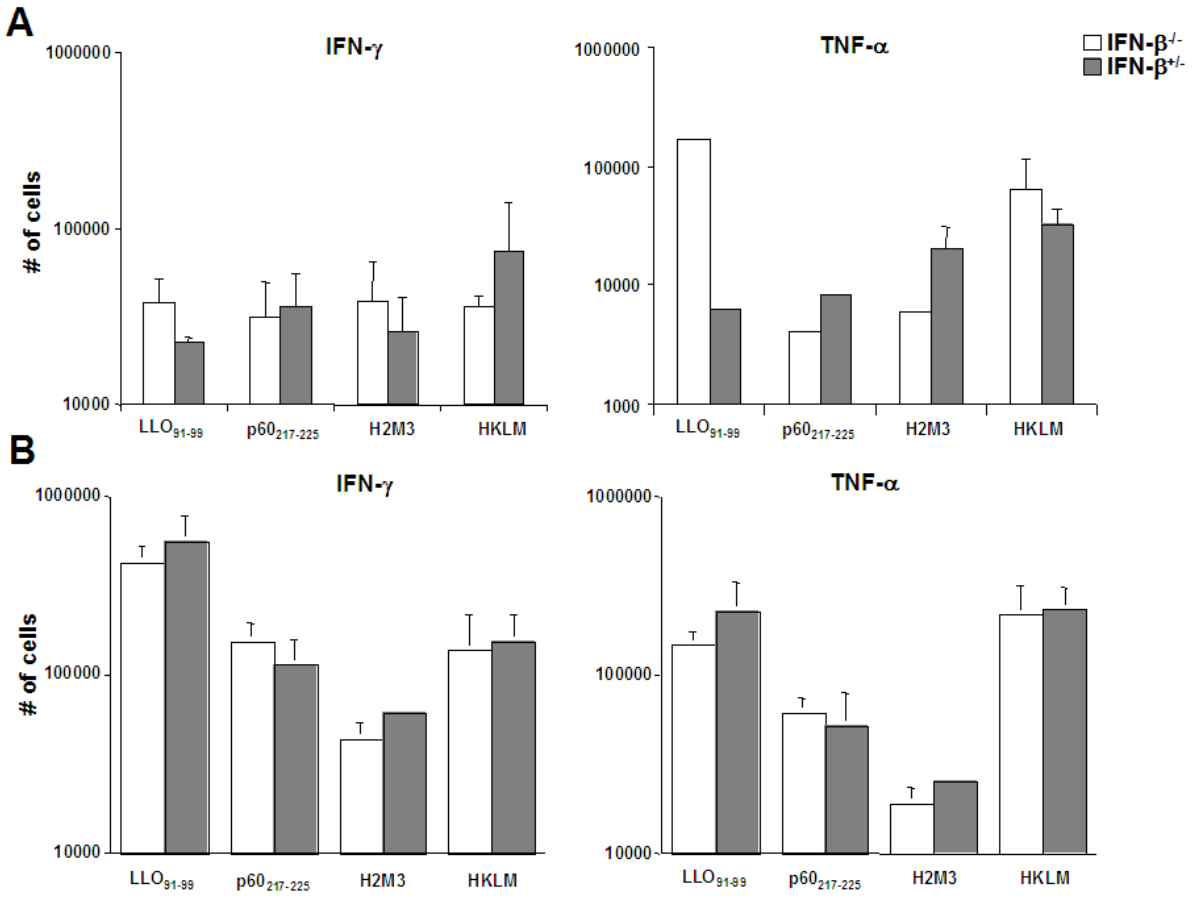


***Fig. 3.17**: **Flow cytometric analysis of intracellular cytokine production by mature CD8^{+}CD62L^{lo} T cells**. **A)** IFN-γ and TNF-α positive cells day 3 after primary immunization. **B)** Day 7 after primary immunization.*

all more mature T cells specific for the various tetramers, although no differences were seen in the amounts of these cells between IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ mice (Fig. 3.16B).
Production of IFN-γ and TNF-α was also determined by intracellular staining at the same time points p.i. Briefly, spleen cells from control and infected mice were restimulated with peptide fragments LLO_{91-99} and $p60_{217-225}$ or with H2M3-peptide or heat killed L.monocytogenes (HKLM). Spleen cells were treated with brefeldin to block cyokine release and intracellular levels of IFN-γ and TNF-α were analysed in mature $CD8^{+}CD62L^{lo}$ T cells. At day 3 both IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ cells readily produced the two cytokines but no significant differences were found between the two populations, although IFN-$\beta^{-/-}$ cells stimulated with LLO_{91-99} seemingly produced more TNF-α than the IFN-$\beta^{+/-}$ cells (Fig. 3.17A). At day 7 p.i. still no differences could be found. Furthermore, there was no variation in production of TNF-α anymore (Fig. 3.17B).

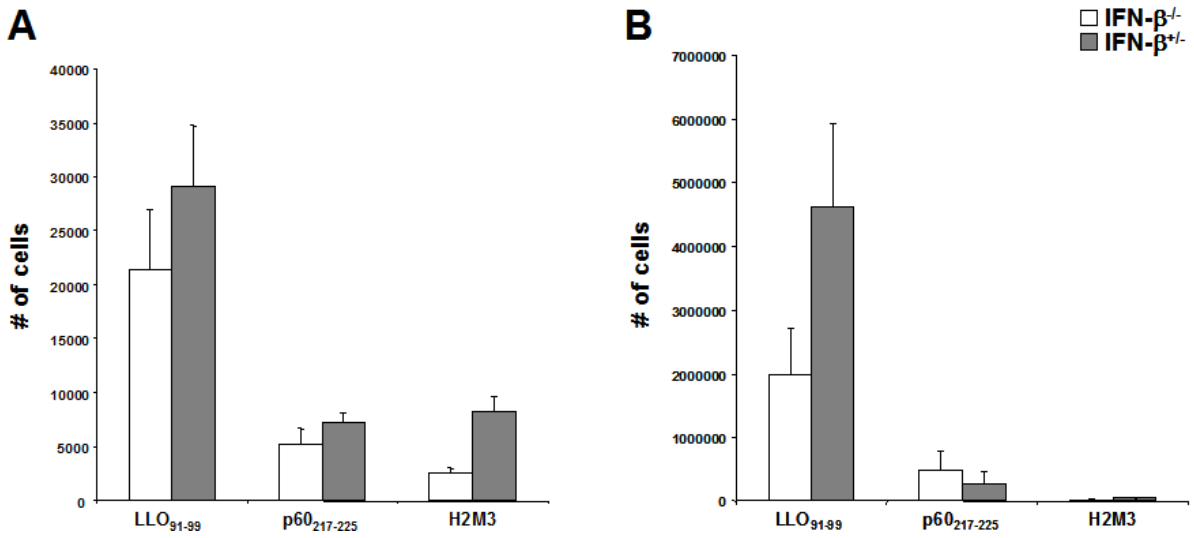


***Fig. 3.18**: Mature $CD8^{+}CD62L^{lo}$ specific for LLO_{91-99}, $p60_{217-225}$ and H2M3-peptide tetramers. A) Day 2 after secondary immunization with L.monocytogenes. **B**) Day 5 after secondary immunization.*

Another group of mice were kept for the analysis of memory responses. 35 days after the initial immunization, the mice were rechallenged with $1x10^5$ *L.monocytogenes*. Again, adaptive immune responses were examined with the same methods as for the primary response, but now instead at days 2 and 5 after the secondary immunization.
Tetramer stainings revealed slightly lower numbers of mature $CD8^{+}CD62L^{lo}$ T cells in IFN-β deficient mice 2 days after secondary immunization (Fig. 3.18A). This difference was even

greater 5 days after secondary immunization, although only for T cells specific for LLO_{91-99}, while T cells specific for $p60_{217-225}$ or H2M3 were very few (Fig. 3.18B). This is consistent with the immunodominant role of LLO_{91-99} (Busch et al., 1998).

Examining the ICS for IFN-γ and TNF-α revealed some differences. At day 2 after secondary immunization there were no observable differences in IFN-γ production, but again TNF-α production was lower in IFN-β deficient cells (Fig. 3.19A), though a large difference was only found in LLO_{91-99} stimulated cells. These differences in TNF-α production were still observable at day 5 after secondary immunization (Fig. 3.19B). Interestingly, also IFN-γ production was now lower in IFN-β deficient cells, though again this difference was only found in the LLO_{91-99} stimulated cells (Fig. 3.19B).

Taken together, only minor deviations could be found in the ability of IFN-β deficient mice to mount an effective primary adaptive immune response to *L.monocytogenes* infection. Also, the memory response was only slightly different when it came to cytokine production.

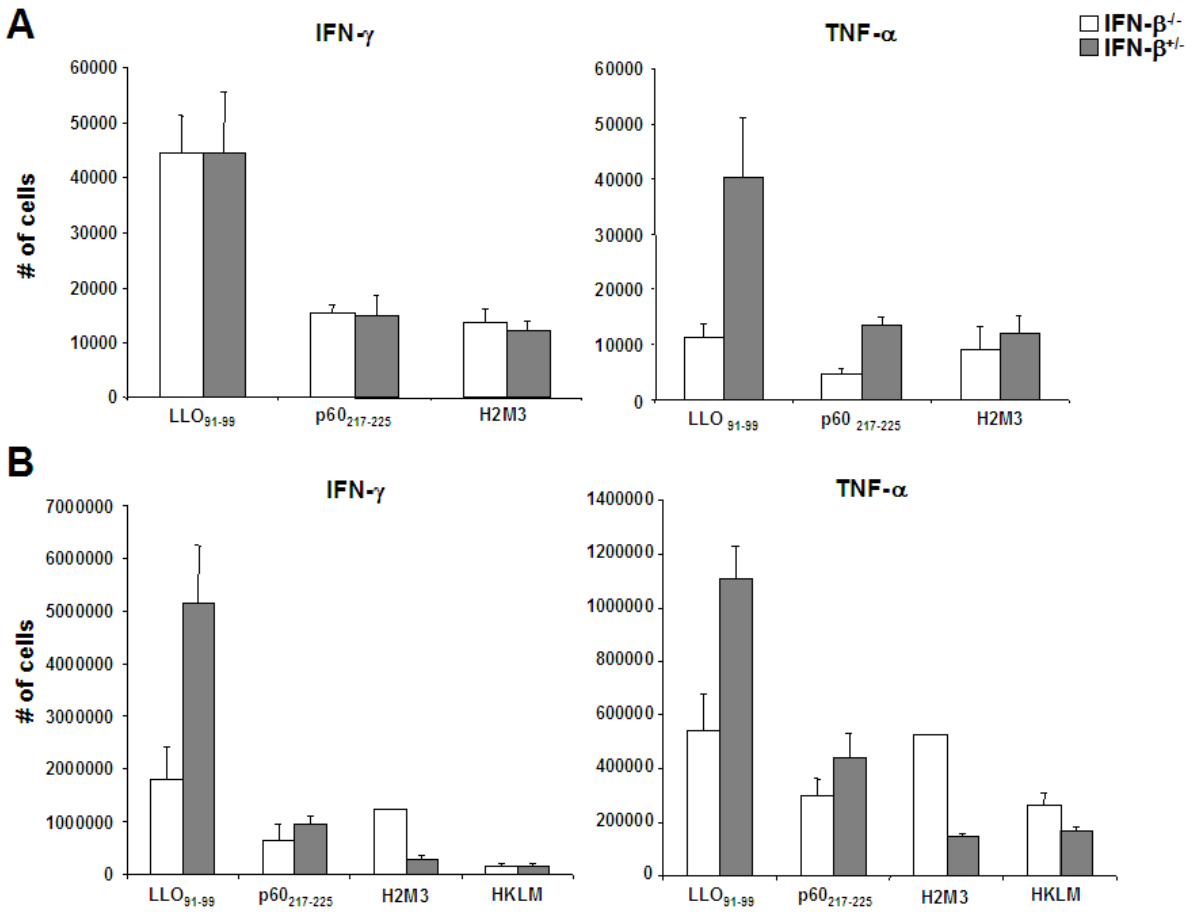


***Fig. 3.19**: **Flow cytometric analysis of intracellular cytokine production by mature $CD8^+CD62L^{lo}$ T cells. A)** IFN-γ and TNF-α positive cells day 2 after secondary immunization. **B)** Day 5 after secondary immunization.*

3.4.4 Analysis of type I IFN expression in splenic cell populations

To further analyse what cell types that *in vivo* express type I IFNs in response to *L.monocytogenes* infection, Balb/c mice were infected with a high dose of *L.monocytogenes* and spleen cells collected and stained before sorted on a cell sorter. RT-PCR analysis was performed for IFN-α4, IFN-non-α4 and IFN-β and is illustrated in Fig. 3.20.

IFN-β and –α4 are often referred to as early responders in the type I IFN system, and are normally required for the subsequent expression of IFN-non-α4s. Interestingly, the splenic macrophage subtypes ERTR-9 and MOMA-1 express IFN-non-α4 without expressing either IFN-β or IFN-α4. Furthermore, this expression is constitutive and does not seem to be induced by *L.monocytogenes* infection. Instead, IFN-β and –α4 are upregulated first 24 h after Listeria infection and only in the ERTR-9 cells, while MOMA-1 exclusively express non-α4 IFNs.

F4/80 positive cells, an additional macrophage subpopulation, only express IFN-α4 24 h after Listeria infection, while conventional $CD11c^{hi}$ DCs upregulate the expression of all type I IFNs 24 h p.i. Interestingly, the so called natural IFN producing pDCs ($CD11c^{int}$/B220) express non-α4s already at 4 h p.i. while IFN-β expression is detected first at 24 h and only weakly so.

Of the other cell types in the spleen, $CD3^+$ T cells express IFN-β and –α4 at 4 and 24 h p.i., while $CD19^+$ B cells constitutively express both IFN-β and –α4. NK cells (Dx5) express of IFN-β at 4 and 24 h p.i. and also IFN-α4 after 24 h. Finally, $Gr\text{-}1^+$ neutrophils very weakly express IFN-β prior to infection, but seem to be induced to express IFN-α4 24 h p.i.

In summary, this panel of cells illustrate the complexity of the type I IFN system. It also emphasizes that IFN-β in certain cases does not need to be the master IFN for an induction of an innate anti-microbial response, and fits well with the observation that IFN-β deficient mice neither are more susceptible nor more resistant to *L.monocytogenes* infection than WT mice.

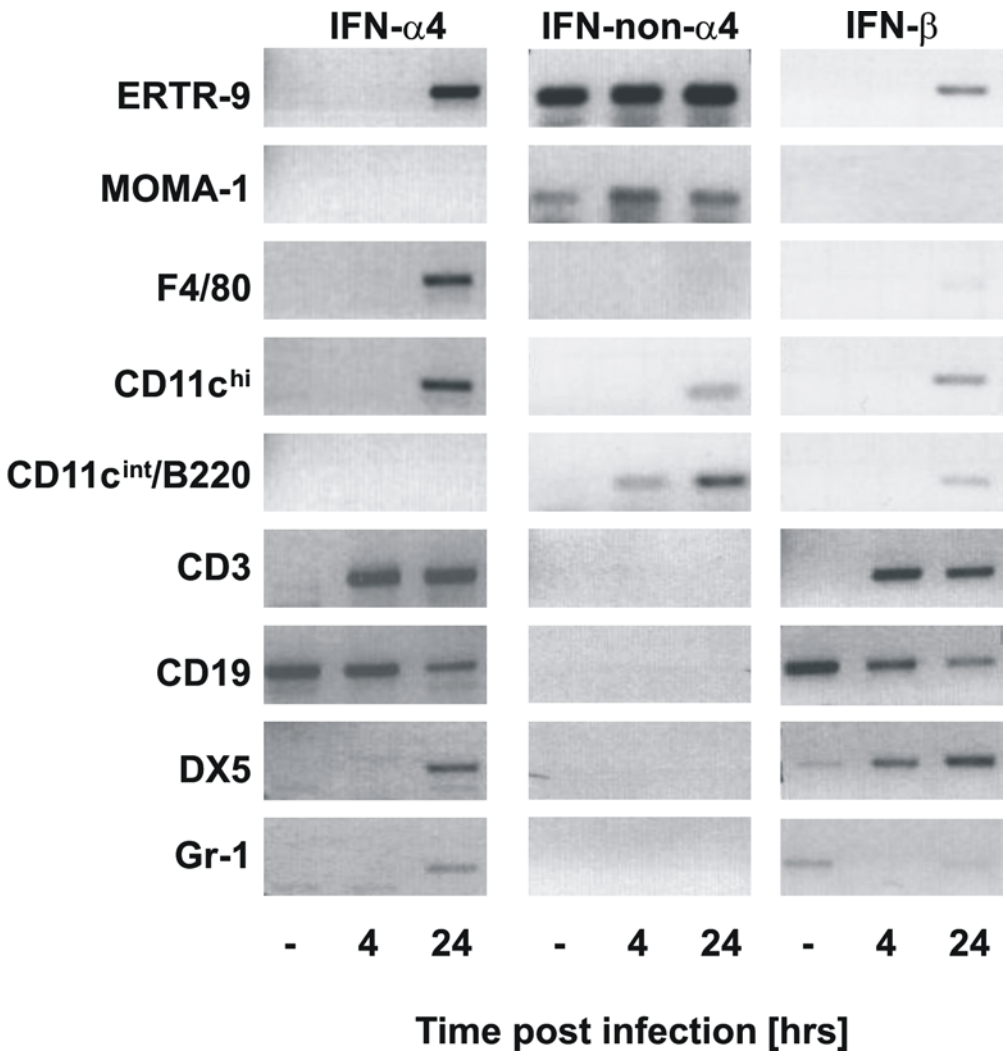


***Fig. 3.20**: **RT-PCR analysis of type I IFN expression in splenic cell populations.** WT BALB/c mice were infected with 5x10^5 L.monocytogenes. At indicated time points post infection, spleens were homogenized and cells from the single cell suspension were sorted by flow cytometry. RT-PCRs were performed as previously described.*

3.4.5 Administration of recombinant IFN-β prior to *L.monocytogenes* infection decreases bacterial load in both liver and spleen

Previous studies have shown that pre-treating mice with recombinant IFN-β can have protective effects in various microbial infections. To investigate whether this was true also in the case of *L.monocytogenes* infection, WT BALB/c mice were injected with different amounts of rmIFN-β 1 h prior to infection with 2x10³ *L.monocytogenes*. A preliminary time course experiment had determined 48 h post infection to be the most suitable time point. Already 100 U rmIFN-β/mouse was a sufficient amount to mount a protective effect in liver (Fig. 3.21 upper panel). This effect increased with higher amounts of administered IFN-β, and

at the highest dose a significantly lower bacterial load was found in liver, as well as in spleen (Fig. 3.21 lower panel).

In summary, high amounts of systemically available IFN-β decreases the bacterial load in liver and spleen after *L.monocytogenes* infection.

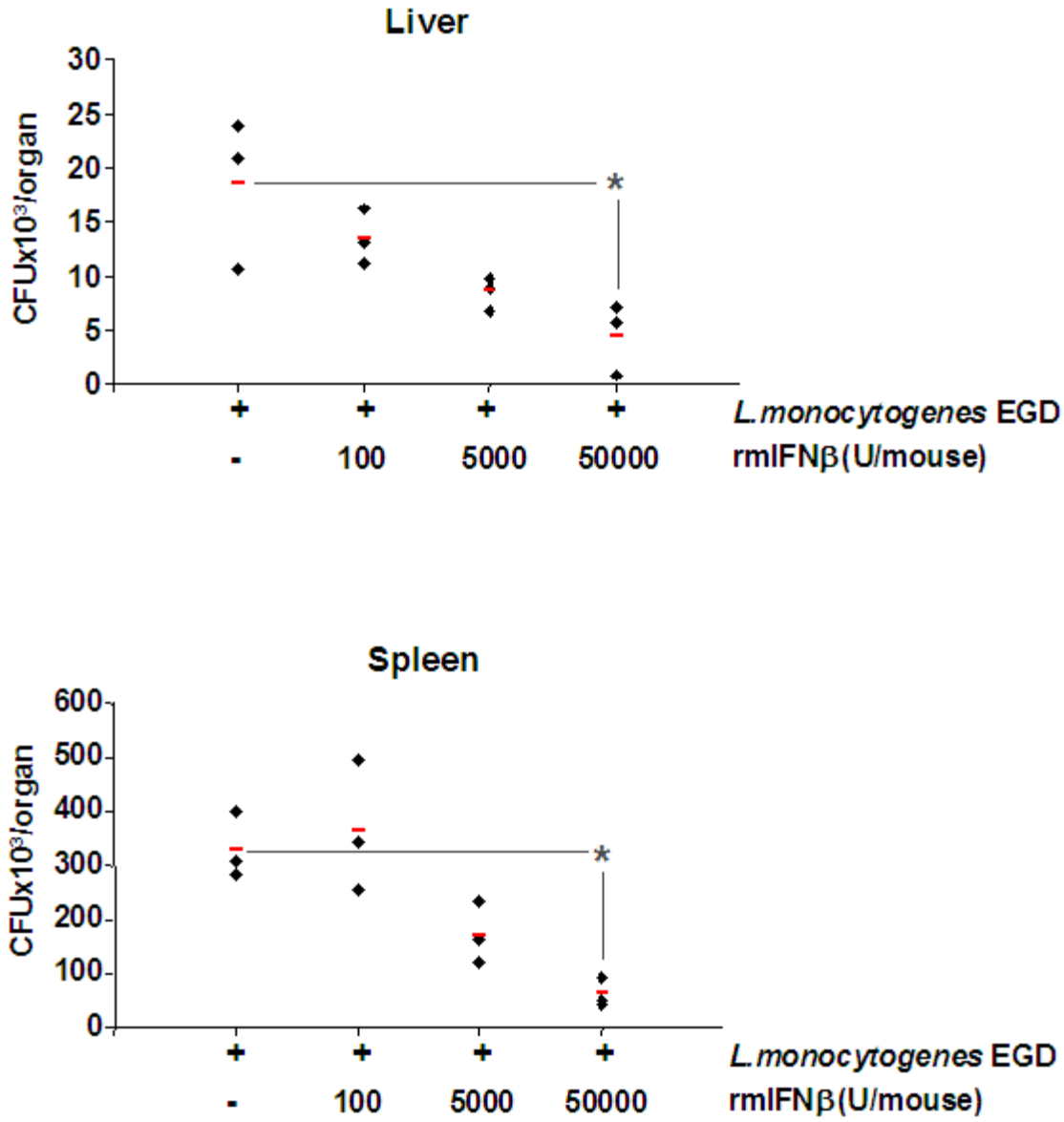


***Fig. 3.21**: **Bacterial loads in liver and spleen determined by CFU from homogenized organs**. Mice were pre-treated with different amounts of rmIFN-β 1 h prior to infection with 2x10³ L.monocytogenes. CFUs in liver decreased with increasing amounts of rmIFN-β (upper panel). This pattern is also seen in spleen though is not as dramatic as in the liver at the lowest concentration (lower panel). Only the highest amount of rmIFN-β decreased bacterial load to statistically significant levels. Graphs are taken from one representative experiment with 3-4 mice per group. (*p<0.05)*

3.5 DSS induced intestinal inflammation

IFN-β has been shown to be a mediator in certain types of inflammation, e.g. in the endotoxin shock model (Karaghiosoff et al., 2003). On the other hand, its presence ameliorates the inflammation reaction in other types of inflammation (Theofilopoulos et al., 2004). For instance, IFN-β is at the moment the only approved therapy for multiple sclerosis (Hafler, 2004). To further investigate this dichotomy, an inflammatory bowel disease model, which describes a complex type of disease that might have multiple causes, was selected.

Several models for studying intestinal inflammation in the mouse exists (Pizarro et al., 2003). An accepted and well studied chemical model is the dextran sodium sulfate (DSS) induced colitis, which is suitable for investigating epithelial responses to injury, neutrophil infiltration or other aspects of the acute phase of colitis pathogenesis.

Several proinflammatory cytokines have been shown to play a role in DSS induced colitis (Rogler et al., 1998). Furthermore, IRF-1 has shown to play a protective role in this model (Siegmund et al., 2001) and so has also ICAM-1 deficiency (Bendjelloul et al., 2000).

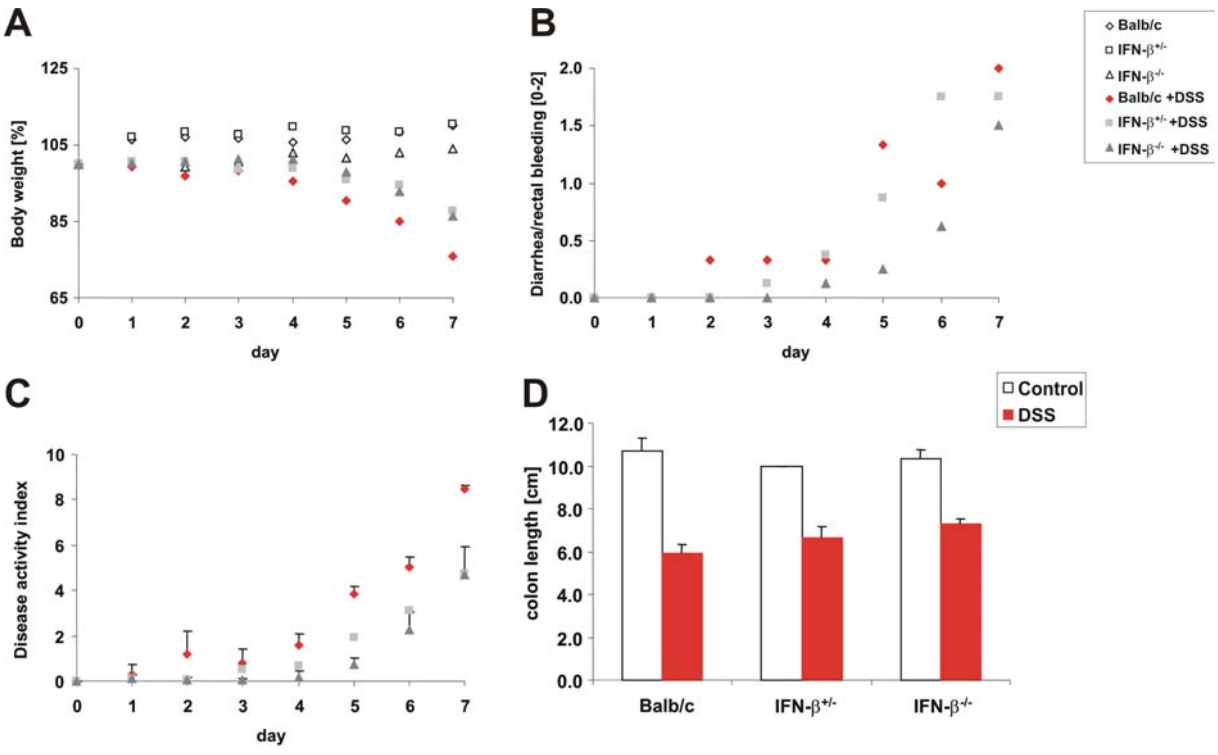


Fig. 3.22: DSS induced colitis in male mice of BALB/c background. *4% of DSS was given in the drinking water over a 7 day period. During this time body weight loss (**A**) and diarrhea/rectal bleeding score (**B**) was monitored. Combining the weight loss in grams with the diarrhea/rectal bleeding score gives the disease activity index (**C**). On day 7, mice were sacrificed and the length of their colons was measured (**D**). In summary, WT mice show a quicker onset of colitis symptoms and also a more severe disease progression.*

A recent study illustrated the requirement of TLRs for intestinal homeostasis (Rakoff-Nahoum et al., 2004), and that several different TLR deficient mice were more susceptible to DSS induced colitis as compared to WT mice. These molecules can all be induced by or induce type I IFNs, making a compelling case for testing the eventual role of IFN-β in the DSS induced colitis model.

3.5.1 Lack of IFN-β ameliorates DSS induced colitis in male BALB/c mice

The initial DSS experiments were conducted on male mice on the BALB/c background, and compared the reactions of two different DSS concentrations in the drinking water, 3% and 5%. The 3% group showed relatively mild symptoms, while the 5% group developed a more severe disease progression were mice in all groups died from day 5 and onwards. 4% DSS drinking water was therefore decided upon as a suitable concentration to proceed with.
Body weight loss was monitored during the duration of the experiment (7 days). WT mice started losing weight earlier than both IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ mice (Fig. 3.22A), and this difference was maintained throughout the remainder of the experiment. Also when scoring for rectal bleeding and diarrhea, WT mice had an earlier onset of symptoms and showed more severe symptoms than both IFN-$\beta^{+/-}$ and IFN-$\beta^{-/-}$ mice by the termination of the experiment (Fig. 3.22B).
Combining weight loss with the diarrhea/rectal bleeding score into the disease activity index (DAI) underlines the earlier onset of symptoms and the more severe progression of disease in WT mice (Fig. 3.22C). Interestingly, IFN-$\beta^{+/-}$ mice had an onset and disease progression more reminding of IFN-$\beta^{-/-}$ mice than of WT mice.
The final read out is the colon length at day 7 of DSS treatment, as inflammatory conditions cause the length of the colon to decrease. WT mice had the shortest colons, IFN-$\beta^{-/-}$ the longest, while IFN-$\beta^{+/-}$ mice had lengths falling in between those of the other two groups (Fig. 3.22D). Also this result demonstrates that WT mice aquire a more severe colitis than mice lacking IFN-β.

3.5.2 Female mice on BALB/c background are overall less susceptible to DSS induced colitis

To investigate eventual gender differences, a 4% DSS dose was also given WT and IFN-$\beta^{-/-}$ female mice on the BALB/c background. As not much happened in ways of disease progression during the first two days of DSS administration, the body weight loss and

diarrhea/rectal bleeding scoring was monitored from day 3 and onwards. Compared to male mice, the females are less affected by the DSS treatment. Body weight loss is less severe in both WT and IFN-$\beta^{-/-}$ mice (Fig. 3.23A), while diarrhea/rectal bleeding is detected later and is overall less severe than in males (Fig. 3.23B). However, the observation that WT mice had an earlier onset and a quicker disease progression than IFN-$\beta^{-/-}$ mice was consistent also in female BALB/c mice (Fig. 3.23A-C). Also here, colon shrinkage was more severe in WT mice than in IFN-$\beta^{-/-}$ mice (Fig. 3.23D).

3.5.3 Analysis of T cell subpopulations in mesenteric lymph nodes of DSS treated mice

It was previously demonstrated that expression of CD69, an early activation antigen on T cells, and the expression of CD25, the IL-2Rα chain, on CD4 cells of the mesenteric lymph

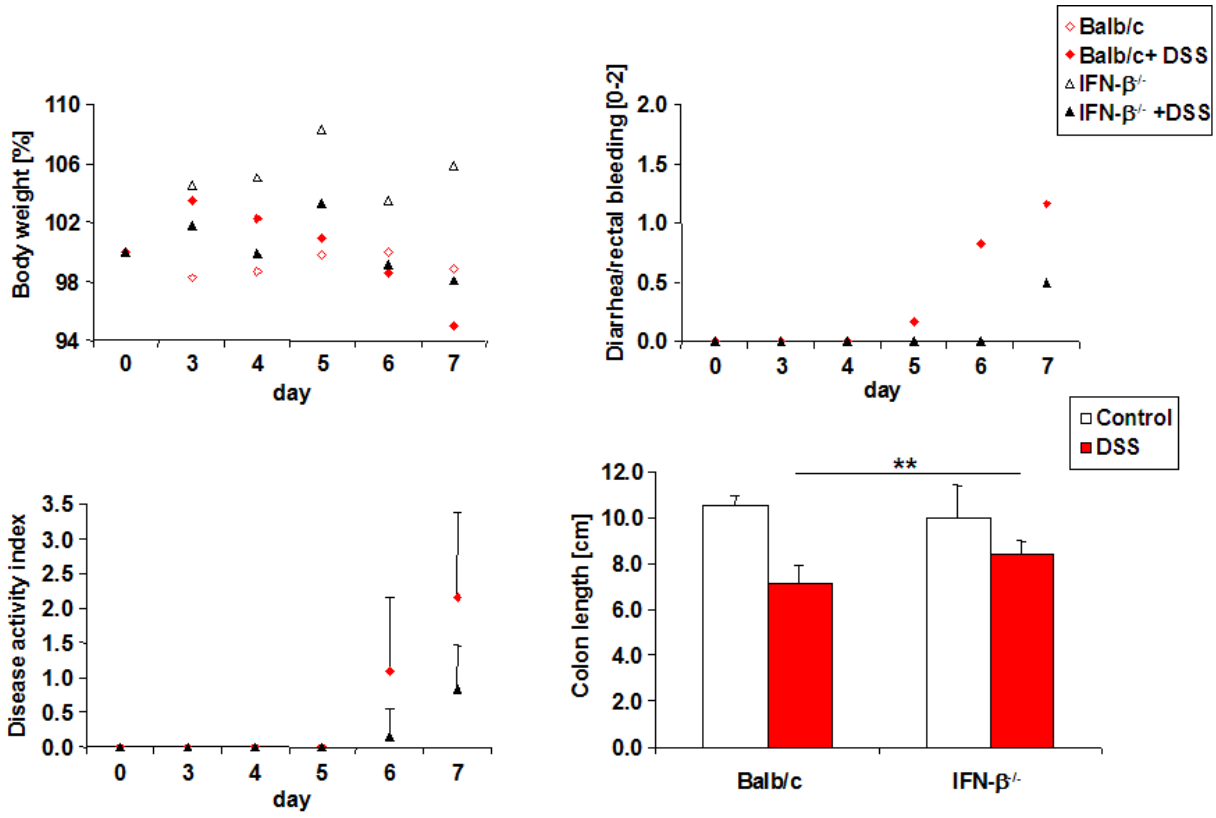


Fig. 3.23: DSS induced colitis in female mice of BALB/c background. *4% of DSS was given in the drinking water over a 7 day period. During this time body weight loss (**A**) and diarrhea/rectal bleeding score (**B**) was monitored. Combining the weight loss in grams with the diarrhea/rectal bleeding score gives the disease activity index (**C**). On day 7, mice were sacrificed and the length of their colons was measured (**D**). In summary, WT mice show a quicker onset of colitis symptoms and also a more severe disease progression. (**p<0.01)*

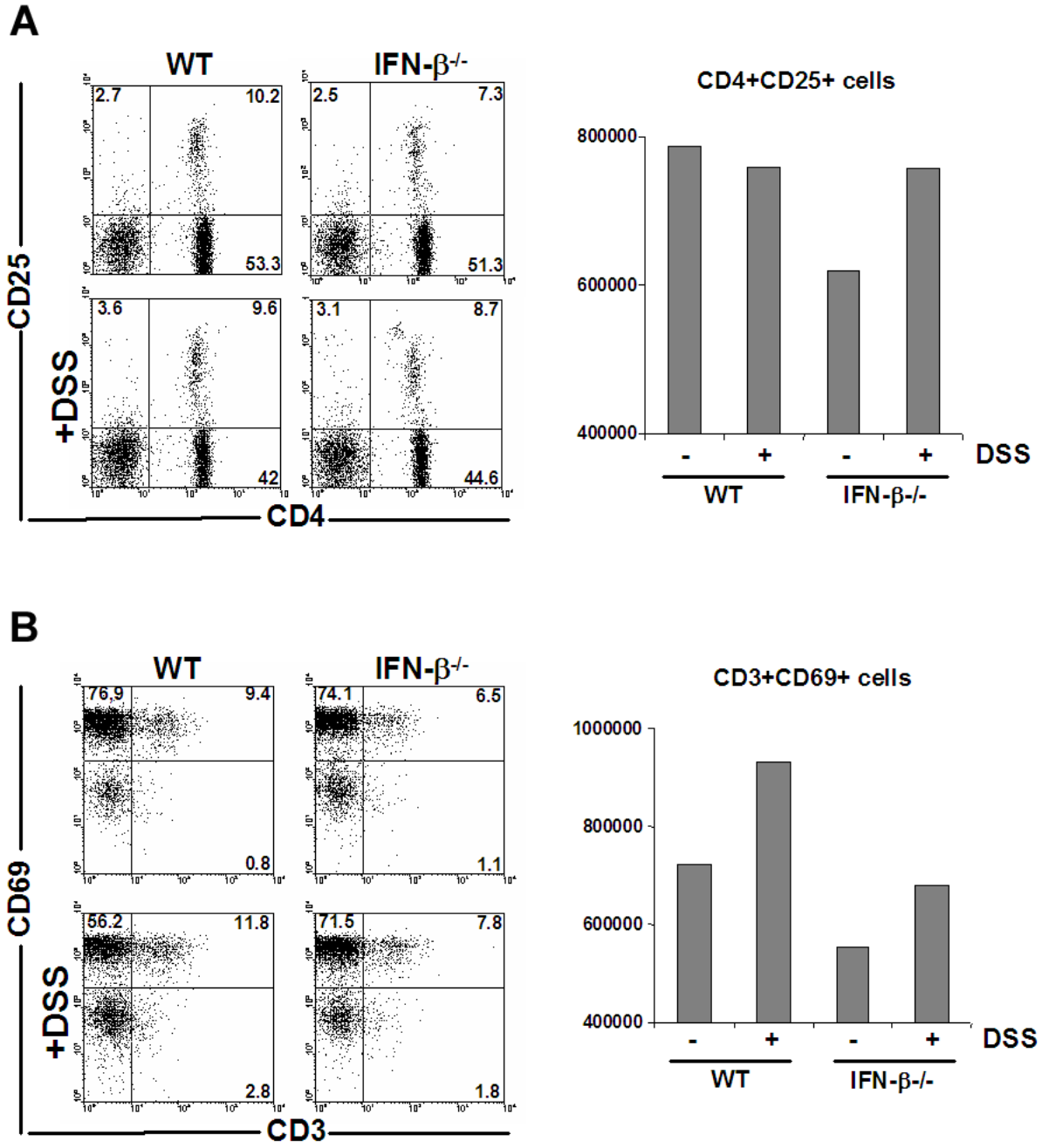


***Fig. 3.24**: **Flow cytometric analysis of CD4 CD25 and CD3 CD69 expression on MLNs during DSS induced colitis**. MLNs were isolated from control and from DSS treated mice on day 7 after treatment started. Numbers in dot plots of flow cytometry indicate percentage of stained cells and graphs on right show total numbers of double positive cells in the MLNs. **A)** CD4+CD25+ cells are more abundant in WT mice and their numbers do not increase after DSS treatment. In IFN-β deficient mice numbers of CD4+CD25+ cells increase to the same level found in WT mice after DSS treatment. **B)** CD3+CD69+ cells are less in IFN-β deficient mice, though numbers increase in both WT and IFN-β-/- mice after DSS treatment. The total number of CD3+CD69+ cells in DSS treated IFN-β-/- mice reach the levels of WT untreated mice. Staining is done once with one mouse per group.*

nodes (MLN) was associated with increased disease activity in the DSS model (Siegmund et al., 2001). An initial experiment was performed to investigate whether these differences could be found also in IFN-$\beta^{-/-}$ mice. Therefore, MLNs were isolated on day 7 after DSS treatment and CD3 and CD4 cells analyzed for their expression of CD69 and CD25 respectively.
The total number of $CD4^+CD25^+$ cells, known to include regulatory T cells, were lower in the IFN-$\beta^{-/-}$ mouse than in the WT mouse. Though, while numbers of $CD4^+CD25^+$ remained the same in the WT mouse after DSS treatment, there was an increase of $CD4^+CD25^+$ cells in the IFN-$\beta^{-/-}$ mouse after DSS treatment (Fig. 3.24A).
The numbers of $CD3^+CD69^+$ were also lower in the IFN-$\beta^{-/-}$ mouse compared to WT, both in control and in DSS treated mice, though both DSS treated WT and IFN-$\beta^{-/-}$ mice increase their number of $CD3^+CD69^+$ cells (Fig. 3.25B).
These initial results need validating but hint that the difference in disease severity is not due to the activation of T cells or the activity of regulatory T cells.

3.5.4 Female C57Bl/6 mice are more susceptible to DSS induced colitis

Finally, female mice on the C57Bl/6 background were administered 4% DSS in the drinking water for 7 days. C57Bl/6 mice are often more prone to inflammatory disease, and this was also the case in DSS induced colitis. Overall, these mice were more severely affected by the treatment than the BALB/c counterparts, as illustrated in Fig. 3.25, and all read outs were more pronounced than in previous animals. However, also on this background IFN-β-/- mice had a less severe disease progression and weight loss was not as rapid and dramatic (Fig. 3.25A). Diarrhea/rectal bleeding was hardly noticeable (Fig. 3.25B) and total DAI was markedly lower than in WT mice (Fig. 3.25C). Colon shrinkage was also not as dramatic (Fig. 3.25D). Taken together, these data show that C57Bl/6 is a more compelling background for studying the effects of IFN-β in DSS induced colitis as disease progression is clearer and the differences between WT and IFN-β-/- larger and more clear-cut.

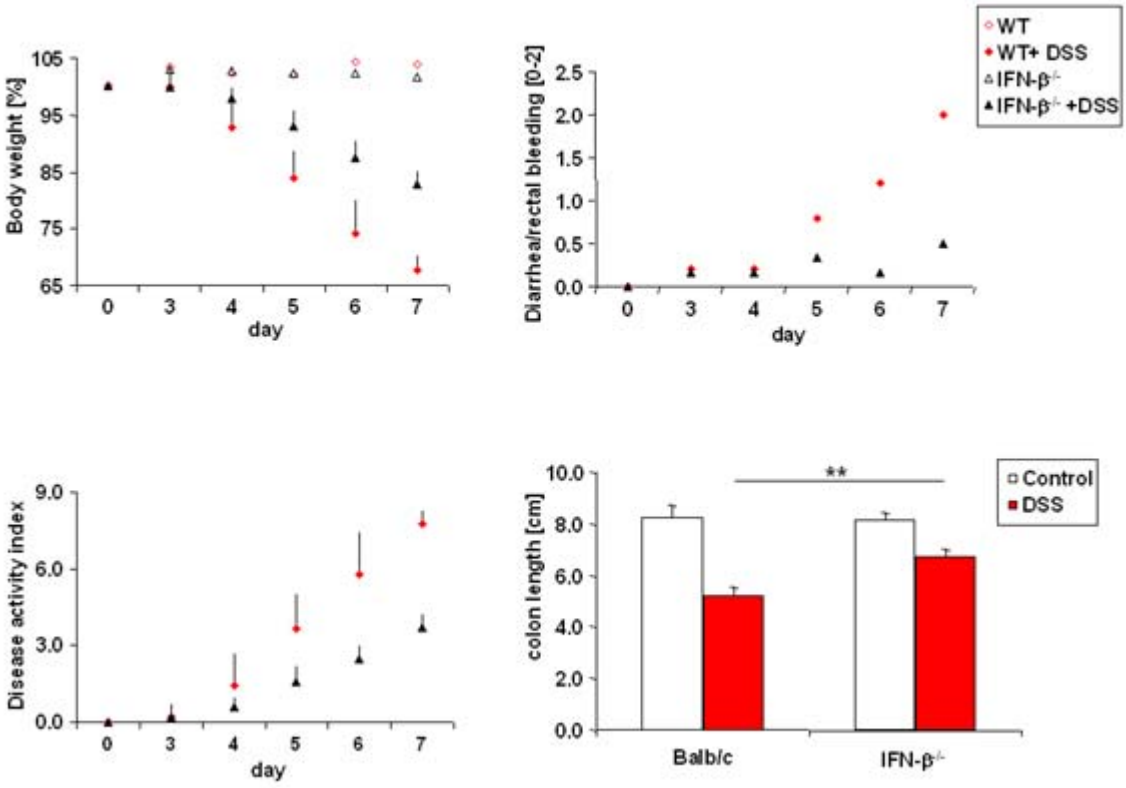


Fig. 3.25: DSS induced colitis in female mice of C57Bl/6 background. *4% of DSS was given in the drinking water over a 7 day period. During this time body weight loss (**A**) and diarrhea/rectal bleeding score (**B**) was monitored. Combining the weight loss in grams with the diarrhea/rectal bleeding score gives the disease activity index (**C**). On day 7, mice were sacrificed and the length of their colons was measured (**D**). In summary, WT mice show a quicker onset of colitis symptoms and also a more severe disease progression. (**p<0.01)*

4 Discussion

The complexity of the IFN system was well known at the start of this project. Data accumulated during this work show that the complexity is even more pronounced than expected. For instance, little attention was previously paid to the hierarchy of type I IFNs in different cell types or the role of type I IFNs in initiation of proliferation. Therefore, the aspects of type I IFNs that were elaborated in the present work should help explain some of the phenotypes that were encountered during work with IFN-$\beta^{-/-}$ or IFNAR$^{-/-}$ mouse lines, or should initiate additional experiments for a broader understanding of the IFN system and its impact on host defences and disease.

4.1 Analysis of the early kinetics and hierarchy of type I IFNs in virus infected fibroblasts

Previous studies have reported that IFN-β and -α4 act as immediate early genes after virus induction (Juang et al., 1998; Sato et al., 2000). This quick burst of secreted type I IFN induces an auto- and paracrine loop that signals via the IFN receptor, leading to activation of the Jak-Stat pathway and expression of IFN stimulated genes (ISGs). Among these ISGs is IRF-7, which binds to the promoter of both the immediate early response genes (IFN-β and -α4) and to the promoters of the delayed IFN-non-α4s, the consequence being a second, enhanced burst of type I IFN secretion (Levy et al., 2002; Marie et al., 1998).
In this work, additional levels of hierarchy of type I IFN responses to virus infection have been examined (Erlandsson et al., 1998). The role of IFN-β as an essential component for a positive feedback loop regulating the expression of IFN-α in mouse fibroblasts has been determined. The absence of detectable IFN-α expression or production in primary IFN-$\beta^{-/-}$ MEF or MAF cultures is likely due to the lack of IRF-7, but not IRF-3, induction in these cells since IRF-3 expression was unaffected by the inactivation of IFN-β (data not shown). The induction pattern of IFN was comparable to the IFN-$\beta^{-/-}$ derived cell cultures, supporting the necessity of IFN receptor signalling in the regulation and hierarchy of IFN expression following SeV infection. Interestingly, neither IFN-α4 nor IFN-non-α4s were detected in IFNAR$^{-/-}$ fibroblasts. These findings are in agreement with an essential role of IFN-β in efficiently triggering the type I IFN cascade.
Immortalization of fibroblasts using the SV40 large-T antigen modified the expression pattern of type I IFNs in the IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ fibroblasts, and IFN-α4 mRNA was now readily

detected 6 h after virus infection. Despite of this, no IFN-non-α4 was detectable in IFN-$\beta^{-/-}$ cells although IRF-7 transcription was induced in immortalized MAFs. One possible explanation for this observation is that IRF-7 was expressed, but not phosphorylated, in the transformed cells and therefore requires additional signals for its activation in these cells. The second more likely possibility is that IRF-7 activity in the absence if IFN-β is delayed, and that IFN-non-α4s are induced after termination of the experiment. This might indicate another temporal hierarchy of type I IFNs, where IFN-β can be designated as immediate early, IFN-α4 as early and the non-α4s as late induced type I IFNs, and is in agreement with the observation made using WT fibroblasts.

The IFN gene expression determined by PCR was confirmed by IFN-bioassays to corroborate production of the mature cytokine after virus induction. Only primary fibroblasts from WT mice showed enhanced secretion of type I IFNs. In immortalized fibroblasts, levels of secreted type I IFN were generally higher compared to levels in primary fibroblasts. This might be due to an increased expression of IRF-7 already in the uninfected state, either because IFN-β is to some extent spontaneously produced in these cells, or because immortalization might facilitate IRF-7 expression through constitutive activity of signalling pathways in the transformed cellular environment. However, the overall theme of dominance of IFN-β in the regulation of type I IFN responses to viral infection were still observed in the immortalized fibroblasts.

Also, measuring the virus loads in IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ fibroblasts again highlighted the necessity for an intact feedback loop. In both primary and immortalized MEFs, the virus loads in WT cells was lower than in the fibroblasts from IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ mice. This would be expected in a setting where not enough type I IFN is produced to stave off a virus infection. The reason underlying the central role for IFN-β in the regulation of IFNα responses is still not clear. A higher affinity of this cytokine to its receptor has been suggested (Deonarain et al., 2002; Morin et al., 2002; Platanias et al., 1996a). In agreement with the special role of IFN-β, additional signalling pathways have been suggested for this cytokine. Cells with impaired components of the IFNAR signalling pathway have been reported to respond differentially to IFN-β and IFN-α (Mogensen et al., 1999).

Why immortalization allows the direct induction of IFN-α4 is also puzzling. Several hundred genes are differentially expressed upon immortalization (May et al., 2004), but no obvious transcription factor responsible for the findings in this work could so far be identified. Nevertheless, comparative analysis of primary and immortalized fibroblasts, as employed

here, should enable a more rigorous analysis of candidate transcription factors that are differently expressed in primary and immortalized fibroblasts and, more importantly, are specific to key control elements in type I IFN genes.

In summary, immortalization of fibroblasts changes the gene expression pattern of type I IFNs to virus infection. Nevertheless, IFN-β is still essential for an efficient expression of type I IFNs. This dominance of IFN-β might also exist in cell types other then fibroblasts, thus, explaining the phenotype of IFN-$\beta^{-/-}$ mice with regard to several infection and inflammation models. Interestingly, this hierarchy of type I IFNs may therefore be altered in dendritic cells and macrophages in the *in vivo* setting (Levy, 2002) and unpublished).

4.2 Influence of IFN-β on the initiation of fibroblast proliferation

IFN-α has for nearly twenty years been used to induce regression of human neoplasias. The IFNs might mediate anti-tumorigenic effects either in indirect or direct ways. Modulating immune and anti-angiogenic responses could be an indirect way, while a more direct way might be via affecting the tumor cells' capacity to proliferate and differentiate. Both these effects of IFNs results from the induction of ISGs which are the primary effectors of the IFN mediated biological responses. Microarray studies in different cell lines treated with IFN have identified more than 300 induced genes (Chawla-Sarkar et al., 2003), and the sheer volume of induced genes has made it difficult to attribute the effects of IFNs to particular gene products, although quite a few have been identified to be involved in mediating the antiproliferative effects of type I IFNs. Cell cycle kinases are a group of genes generally inhibited after treatment with IFN-α. This inhibition leads to a suppression of pRb phosphorylation, therefore slowing progression into S phase. Also, PKR (Chong et al., 1992; Dever et al., 1993; Koromilas et al., 1992) and RnaseL (Zhou et al., 1998) have been shown to be important mediators of the type I IFN's antiproliferative effects.

Fibroblasts from IFN-$\beta^{-/-}$ mice show earlier onset of cell proliferation compared to fibroblasts from WT mice after seeding at low densities. The observed retardation in growth in WT fibroblasts can either be due to a constant production or an induced production of IFN-β. It is known that low levels of IFN is detected in human tissue also without stimuli (Taniguchi et al., 2001), though it has also been shown that PKR can exhibit activity related to physiologic stress in the absence of dsRNA (Ghosh et al., 2000). Thus, it is likely that manipulation during harvesting and seeding, together with a loss of cell contact, might act as a

physiological stress signal that induces IFN-β production, as the differences in growth initiation between WT and IFN-$\beta^{-/-}$ fibroblasts increase as cell denstities decrease.
The level of produced IFN-β has to be very low, as no type I IFN in the supenatants were detected with an IFN-bioassay (data not shown). However, the production appeared to be high enough to revert the growth curve of IFN-$\beta^{-/-}$ fibroblasts when adding WT fibroblasts with the IFN-β producing capacity intact. This might also be taken as proof that the active compound responsible for growth retardation is IFN-β. Nevertheless, for a compelling argument, the activity of IFN-β should be inhibited with the use of an antibody. In this case, WT fibroblasts should have a growth curve similar to that of IFN-β deficient cells.
IFNAR$^{-/-}$ fibroblasts should have a growth increase comparable to or even greater than the IFN-$\beta^{-/-}$ fibroblasts, since their type I IFN signalling pathway is completely disrupted. This would eliminate any effects that IFN-α might have on the growth rates of fibroblasts. As the IFNAR$^{-/-}$ fibroblast growth curve indeed is comparable to the one of IFN-$\beta^{-/-}$ fibroblasts, or even slightly decreased, IFN-α does not seem to be very important in this context. This could be due to the intrinsic hierarchy of type I IFNs where, in fibroblasts, IFN-β is required for the efficient transcription and secretion of the IFN-αs as shown in the previous section.
Cell cycle analysis was employed to compare cell cycle profiles of WT and IFN-β deficient fibroblasts. However, no clear picture emerged as to where in the cell cycle IFN-β might act to inhibit initiation of proliferation in WT fibroblasts. Adding exogenous IFN-β also did not alter the profiles in any dramatic way for the duration of the observation period. This might be taken as an indication that the lack of IFN-β does not exert an imprinting effect within the confluent phase of preceding cell cultures. Though one caveat with the cell cycle analysis, as with the CFSE-analysis, was that high density cultures were used in order to have enough cells for the analysis. As previously stated the density has an effect on the initiation of proliferation, therefore these studies need to be repeated in low density cultures, which was not possible within the given time frame of this work.
Furthermore, the absence of an increase of cell numbers despite measurable proliferation in MAFs might be attributed to cells undergoing apoptosis, and should also be investigated in more detail.
In summary, WT fibroblasts show a delayed cell density dependent initiation of proliferation compared to IFN-$\beta^{-/-}$ and IFNAR$^{-/-}$ fibroblasts. This is either due to an induced production of IFN-β due to loss of cell contact, to a continuous spontaneous production of IFN-β or to

effects IFN-β might exert in the preceding confluent culture. The secreted amounts of IFN-β are low and undetectable in an IFN-bioassay, and the effects are not due to a clonal phenomenon.

Further studies should be done to bring these experiments to a conclusive end. One possibility that exists is to add antibodies against IFN-β to the fibroblast cultures as previously mentioned. In addition, a read out system sensitive enough to detect also very low levels of IFN-β in supernatants should be sought. One possibility that exists is to look for STAT-1 translocation into the nucleus by employing immunocytology or cell lines over expressing STAT-1 coupled to a fluorescent marker. Another possibility is to examine tyrosine phosphorylation of STAT-1, though also in this case a sensitive enough responder cell line must be found.

The novel finding that IFN-β delays cell density dependent initiation of proliferation has interesting *in vivo* implications. Thus, IFN-β might delay cellular growth after tissue injury and during wound healing. These processes might be improved by blocking local IFN-β action. Similarly, the regeneration of organs, especially. liver, could be problematic since HCV and HBV infections are treated with type I IFNs. Thus, this finding needs to be extended to *in vivo* models of tissue regeneration and growth.

4.3 Effect of IFN-β deficiency on bone marrow derived APCs

The adaptive immune response is dependent on costimulatory surface molecules, e.g. CD40, CD80 and CD86, which are expressed on the surface of APCs. These molecules help to generate a sufficient response within T cells that are exposed to antigen on MHC class-I or –II molecules. A recent study demonstrated that this upregulation can be induced by LPS and dsRNA in both TRIF-dependent and –independent ways (Hoebe et al., 2003), suggesting an involvement of IFN feed back loops. Therefore it was of interest to study the role of IFN-β in antigen presenting cells (APCs), in the context of UCM and the capacity of APCs to activate T cell proliferation, after stimulation with virus and different PAMPs.

Macrophages and DCs were differentiated from bone marrow of WT and IFN-$\beta^{-/-}$ mice and stimulated with LTA, LPS, poly I:C and SeV for 24 hours. LTA, which is recognized by TLR-2, does not stimulate type I IFN secretion in both BMMϕ and BMDC, as expected. LPS did not induce type I IFN in BMDCs, the most likely reason being an absence of TLR-4 expression in these cells. Induction in BMMϕ was found only in WT cells. This absence of type I IFN in IFN-$\beta^{-/-}$ BMMϕs was interesting as both poly I:C and SeV induced type I IFN

secretion in BMMϕ and BMDC. However, total amounts for both BMMϕs and BMDCs were lower in IFN-$\beta^{-/-}$ cells than in WT cells, highlighting the importance of IFN-β in induction of a complete type I IFN response as previously seen for fibroblasts. Surprisingly, amounts of secreted type I IFN were lower in BMDCs than in BMMϕs, as DCs normally are better equipped for high type I IFN production. This might be explained by the culture conditions chosen for generating BMDCs, as both GM-CSF and IL-4 are known to induce maturation of DCs. Mature DCs are not as easily stimulated and therefore might produce lower amounts of type I IFN.

Differences in upregulation of costimulatory molecules were also found, though only in BMMϕs stimulated with poly I:C. BMDCs were not investigated due to low cell yields, but should in future be included in such studies. In LTA, LPS and SeV stimulated BMMϕs no consistent differences were found and more experiments should be done to clarify this, especially in the case of LPS. Stimulating IFNAR$^{-/-}$ peritoneal macrophages with LPS or poly I:C does not upregulate CD40 or CD86 whatsoever (Hoebe et al., 2003). In contrast, in IFN-$\beta^{-/-}$ BMMϕ this upregulation is only partially affected after poly I:C stimulation, indicating that IFN-β in this setting is redundant.

Since some differences were found in UCM and also in the upregulation of MHC-molecules, the antigen presenting and T cell stimulatory capacity of these APCs was tested. In BMDCs no differences were found, while WT BMMϕs stimulated with LPS and poly I:C had an increased capacity to stimulate T cell proliferation compared to IFN-β deficient cells. This again highlights how different cells types vary in their dependency on the type I IFNs. It also indicates that various stimuli can trigger differential reactions of the IFN system. This is consistent with the recent finding that splenic subpopulations of DC respond differentially to VSV infections *in vivo*.

These findings also might explain the results that were obtained in various infection models. Depending on the route of infection and the cell type targeted by the pathogen differential effects of the lack of IFN-β or the IFNAR were observed (Barchet et al., 2002; Miller et al., 2003; Muller et al., 1994; zur Lage et al., unpublished).

4.4 Role of IFN-β in *L.monocytogenes* infection

In addition to viruses, a large number of microbes and microbial products have been described to trigger production of type I IFNs both *in vivo* and in various cell types *in vitro*

(Bogdan, 2000). Furthermore, injecting recombinant type I IFN can protect mice from various microbial challenges, including *L.monocytogenes* (Fujiki et al., 1988).

However, recently it was shown that disturbing the type I IFN pathway increases resistance to *L.monocytogenes* infection (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). Signalling elicited by type I IFNs after *L.monocytogenes* infection seems to decrease the clearance of bacteria by inducing apoptosis of T cells (Carrero et al., 2004; O'Connell et al., 2004) and the loss of TNF-producing cells (Auerbuch et al., 2004). Type I IFN were previously also shown to decrease the viability of *L.monocytogenes* infected macrophages (Stockinger et al., 2002). Type I IFNs therefore seem to impair the immune defence against *L.monocytogenes*, which is in contrast to their protective function in the innate immune response against viruses. This, together with the reported immunomodulatory effects of type I IFNs (Theofilopoulos et al., 2004), initiated the examination of the susceptibility of IFN-$\beta^{-/-}$ mice to *L.monocytogenes* infection.

WT and IFN-$\beta^{-/-}$ mice infected with a low dose of *L.monocytogenes* show no difference in bacterial loads of spleen and liver. This is in contrast to IRF-$3^{-/-}$ and $IFNAR^{-/-}$ mice, that have significantly lower bacterial loads in both organs after low dose infections and have higher survival rates after high dose infections (Auerbuch et al., 2004; Carrero et al., 2004; O'Connell et al., 2004). Interestingly, pre-treating mice with rmIFN-β lowers bacterial loads in liver and spleen in a dose dependent manner. This indicates that a systemic distribution of IFN-β can exert protective effects in *L.monocytogenes* infections, even though endogenous production of type I IFNs during *L.monocytogenes* infection has detrimental effects on the host. A likely explanation for this is that different effector cells are affected in the two experimental settings. For example, it was shown that IFN-β pre-treatment of *Leishmania major* infected mice enhanced the NK cell cytotoxicity and protected mice against progressive leishmaniasis (Bogdan et al., 2004). On the other hand, mice with a disrupted type I IFN system increase the clearance of bacteria by the inhibition of T cell apoptosis (Carrero et al., 2004; O'Connell et al., 2004) and maintained numbers of TNF-producing cells (Auerbuch et al., 2004). Apparently, IFN-β deficiency does not sufficiently disturb the total IFN-system to cause differences in bacterial clearance in WT or IFN-$\beta^{-/-}$ mice.

Why this is the case most likely depends on what cell type is most prone to be infected by *L.monocytogenes* and how infection initiates type I IFNs. Splenocytes were sorted from BALB/c mice after a high infection dose of *L.monocytogenes* and type I IFN induction was analysed by RT-PCR. Interestingly, different macrophage and DC subtypes express IFN-α

without prior expression of IFN-β. This fits the mentioned bacterial load study, where no differences were found in WT from IFN-$\beta^{-/-}$ mice. One caveat, is of course the use of different infection doses in the two experiments and that the panel of cells expressing type I IFN is only done for WT mice. These experiments should be repeated after both high and low dose infections to further investigate events in the type I IFN system after *L.monocytogenes* infection.

Finally, adaptive immune responses to *L.monocytogenes* infection were investigated. As expected after the bacterial load studies no major differences were found in the primary adaptive response of WT and IFN-$\beta^{-/-}$ mice. Although, IFN-β deficient mice had a slightly better specific killing response at day 5 p.i. As no differences were found earlier or later this result needs to be validated as groups of mice were small and the experiment only repeated twice.

In addition, T cell responses were studied by tetramer- and intracellular cytokine staining. Again, primary responses did not differ between WT and IFN-$\beta^{-/-}$ mice. Interestingly, when a secondary response was induced differences in responses became detectable in later stages of the memory response. On day 5 after the second challenge with *L.monocytogenes* more LLO_{91-99} specific T cells were found in IFN-$\beta^{+/-}$ spleens than in IFN-$\beta^{-/-}$ spleens. Furthermore, IFN-γ and TNF-α production by mature CD8 T cells was also higher in IFN-$\beta^{+/-}$ mice. IFN-γ and TNF-α are essential for the defence against infection with *L.monocytogenes* (Pamer, 2004), and type I IFNs keep activated T cells alive and enhance their proliferation (Marrack et al., 1999), probably explaining this difference. Again, these experiments need to be repeated and in future with WT mice instead of IFN-$\beta^{+/-}$ mice. The reason for this will be discussed more in the next section.

In summary, IFN-β alone is not enough to ameliorate the detrimental effects of the type I IFNs after *L.monocytogenes* infection. Most likely because the IFN-β dominance characteristic of fibroblasts and other cell types, does not exist in the main producers of type I IFNs during Listeria infection, i.e. certain subpopulations of splenic macrophages and DCs.

4.5 DSS induced intestinal inflammation

Type I IFNs have been shown to have both detrimental and beneficial effects on different inflammatory diseases. The use of type I IFNs in treating MS is well known (Hafler, 2004), and IFN-$\beta^{-/-}$ mice thus have an increased inflammatory progression and severity in the murine EAE model (Teige et al., 2003). On the other hand, IFN-β deficient mice are resistant to

endotoxin sock (Karaghiosoff et al., 2003). Since the conditions under which type I IFNs promote or inhibit inflammatory responses remain to be defined, it was of interest to study the disease progression in IFN-β deficient mice in a well studied model of intestinal inflammation.

The initial experiments were carried out in male BALB/c WT and IFN-$\beta^{-/-}$ mice, and IFN-β deficiency ameliorated the disease symptoms. Since gender differences on the impact of autoimmune and inflammatory diseases is well established (Tiidus, 2000), the DSS model was tested in both male and female mice. Interestingly, female mice were less affected than the male mice. Besides hormonal differences, one plausible explanation is the additional stress male mice had to endure as 3-4 males were kept in the same cage. In addition, also C57Black/6 females were tested. This mouse strain has previously been shown to be more susceptible to DSS induced colitis than the BALB/c strain. This was also indeed the case, as female C57Black/6 mice had a disease progression similar to that of male BALB/c mice. Another interesting phenomenon that was observed during these experiments was the disease progression in male IFN-$\beta^{+/-}$ mice. The expectation was that these mice would show a phenotype resembling that of WT mice. Surprisingly, the opposite was correct and the IFN-$\beta^{+/-}$ mice exhibited a phenotype almost exactly resembling the phenotype of IFN-β deficient mice. This could be explained by a mono allelic expression of IFN-β. Thus, the cells producing IFN-β would be less than in the WT mice. However, a more likely reason is that the IFN system is extremely concentration dependent. This would be consistent with the finding that heterozygous IFNAR$^{+/-}$ mice show an intermediate phenotype between WT and homozygous IFNAR$^{-/-}$ mice in a murine SLE model (Goerg et al. personal communication). In summary, IFN-β deficiency ameliorates DSS induced inflammatory bowel disease. The reason for this remains unclear and additional experiments need to be conducted to receive a clue as to what is taking place on a cellular and molecular level. One initial experiment looking at the T cell composition of the mesenteric lymph modes (MLN) was performed, as it had previously been reported that the numbers of activated T cells and T_{reg} cells were changed upon DSS induction (Siegmund et al., 2001). One present caveat is that there is no possibility to distinguish $CD4^+CD25^+$ activated T cells from T_{reg}, and therefore one can only speculate which of these cells would be involved. Thus far, only a single experiment was performed. Far from being conclusive, the result still raises several questions. The biggest question mark is the relevance in analyzing MLN cell composition, as they most probably are not the colon draining lymph nodes. Nevertheless, adoptive transfer experiments might allow to distinguish

the effector function of these T cell populations. Studying the cellular composition of the colon by immunohistology and flow cytometry is another prerequisite for understanding the effect of IFN-β on the inflammation. This needs to be accompanied by analysis of the cytokines that are induced under the different conditions. Combined, these approaches should assist in finding candidate cells and molecules involved in disease progression and in the inflammatory response.

4.6 Conclusive remarks

During the course of this work data were accumulated that added novel aspects to the role of type I IFNs, and especially IFN-β, in certain biological systems. From these data, and results published in the recent past, it can be predicted that type I IFNs play a major role in almost all reactions that involve host pathogen interactions or inflammatory cytokine responses. Some urgent questions to be solved are arising from the present work. For instance, the molecular explanation for the hierarchy in some cells and its abolishment in others will be of high interest. Similarly, comparing other inflammatory bowl disease models for the role of IFN and extending these data to clinical settings will help to see whether IFNs could act as a common drug target. Nevertheless, further aspects are bound to be unravelled when other biological systems are tested for the role of type I IFNs using available or newly generated tools. Thus, the present study has to be considered a step in this direction.

5 Summary

At the start of this work the complexity of the IFN system was well known. The generated data within the project has added to this complexity, though also initiated work that will hopefully broaden the understanding of the type I IFN system in infection and inflammation.
The first part of the work concerned the hierarchy of the type I IFNs in fibroblasts. The dominance of IFN-β for an efficient expression of type I IFNs in fibroblasts after virus infection was confirmed by using primary cells. Immortalized fibroblasts had a modified expression pattern of type I IFNs in IFN-β deficient cells after virus induction, although secretion of type I IFN from these cells remained similar to that of the primary fibroblasts. This dominance of IFN-β might be found in other cell types, but might also be absent, and has profound implications for how certain cells respond to different stimuli.
Another part investigated the influence of IFN-β on cell proliferation. IFN-β was found to delay initiation of proliferation in a cell density dependent manner *in vitro*. This finding could be of importance also *in vivo*, especially in wound healing and in tissue regeneration.
The immunomodulatory role of type I IFNs is also well known. Confusingly, type I IFNs can be both protective and detrimental to the host. Specific cells also differ in their dependence on type I IFN. When bone marrow derived macrophages and dendritic cells were tested for their T cell stimulatory capacity *in vitro*, macrophages exhibited a dependence on IFN-β while dendritic cells did not react differently when IFN-β was absent.
In an *in vivo* bacterial infection model, in which type I IFNs were previously shown to have detrimental effects on the host, lack of IFN-β did not ameliorate the effects of the bacterial infection. This again highlights that some cell types probably do not require IFN-β as a master inducer of type I IFNs, which was also observed when analysing type I IFN expression patterns in spleen cells sorted after infection.
Finally, IFN-β deficient mice were less susceptible to a chemically induced inflammatory bowel disease model, again illustrating a setting in which type I IFN production is detrimental to the host.
Taken together, this work highlights the importance of mapping how different cells types involved in immunological reactions react to different stimuli concerning type I IFN induction. It will be important to find out how and why different cells have different type I IFN hierarchies. This might lead to explaining the different phenotypes found in different inflammatory models. Furthermore, IFNs are commonly used as therapies against multiple

sclerosis and hepatitis infections, and this work indicates possibilities where inhibiting the IFNs, e.g. by antibodies, might be of clinical interest.

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Danksagung

Die vorliegende Arbeit entstand an der Gesellschaft für Biotechnologische Forschung (GBF), Braunschweig, in der Arbeitsgruppe Molekulare Immunologie.

Ich danke dem Mentor dieser Arbeit, Herrn Prof. Dr. Jürgen Wehland, für die Begleitung des Promotionsverfahrens, Herrn Prof. Dr. Stefan Dübel für die Übernahme des Koreferates sowie Herrn Prof. Dr. Norbert Käufer für seine Bereitschaft, als Prüfer zur Verfügung zu stehen.

A very special thank you goes to Dr. Siegfried Weiß, in whose labgroup this work was done, for excellent supervision and discussions, especially about the project in particular and science in general but also about the incompetency of Eintracht Braunschweig.

A major contribution to the completion of this work was made by the Molecular Immunology group, providing an excellent working environment. Special mention goes to Dr. Stefan Lienenklaus for always being there to discuss with and for the "fun" of force feeding mice with OVA-beads. Also a huge thank you to Dr. Jadwiga Jablonska for tirelessly injecting mice and thereafter weighing organs with me. Finally, thank you Regina Lesch and Susanne zur Lage for excellent technical assistance and for keeping us lab rats in order!

Furthermore, the collaborators can not go unthanked. Dr Hansjörg Hauser and Dr Peter Müller at the Dept. Gene Regulation and Differentiation, GBF; Dr. Rainer Zawatzky, DKFZ, Heidelberg; and Prof. Dr. Dirk Busch and Kathrin Linkemann at the TU Munich.

Finally, I must thank my family for always supporting me through thick and thin. I love you!

And to all my friends around the world for making life special and enjoyable.

Ett särskilt tack riktas till "svenskarna" i Braunschweig. Utan vårfester, midsommarfirande, kräftskivor och julbord hade arbetet aldrig flutit på som det gjort.

Zum schluss ein riesen dank an die Jungs von SV Melverode-Heidberg – „Forever number one!".